

SHORT REPORT

Mutation screening of brain-expressed X-chromosomal miRNA genes in 464 patients with nonsyndromic X-linked mental retardation

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MiRNAs are small noncoding RNAs that control the expression of target genes at the post-transcriptional level and have been reported to modulate various biological processes. Their function as regulatory factors in gene expression renders them attractive candidates for harbouring genetic variants with subtle effects on IQ. In an attempt to investigate the potential role of miRNAs in the aetiology of X-linked mental retardation, we have examined all 13 known, brain-expressed X-chromosomal miRNAs in a cohort of 464 patients with non-syndromic X-linked MR and found four nucleotide changes in three different pre-miRNA hairpins. All the observed changes appear to be functionally neutral which, taken together with the rarity of detected nucleotide changes in miRNA genes, may reflect strong selection and thus underline the functional importance of miRNAs.

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MiRNAs constitute an abundant class of small noncoding RNAs that regulate gene expression on the messenger level through sequence-specific base pairing. Recently, several miRNAs have been implicated in a variety of biological processes including developmental timing, programmed cell death, haematopoietic lineage differentiation, insulin secretion, and brain morphogenesis.^{1–5} It is therefore conceivable that defects in miRNAs play an important role in human genetic diseases.

Mental retardation (MR) is a very heterogeneous disorder with a strong influence of hereditary factors. Males are significantly more often affected than females, which suggested an important role of X-linked genes.⁶ This has been borne out by the identification of causative mutations in >20 protein-coding genes for nonsyndromic, or 'pure', X-linked MR (XLMR),⁷ but recent epidemiological studies have shown that disease-causing mutations underlying X-linked recessive MR can explain at most half of the male excess observed.^{8–10} Mandel and Chelly¹¹ have speculated that part of the male excess might be due to the existence of X-chromosomal modifiers with more subtle effects on the IQ. This model would also explain the finding that mutations in the ARX gene are significantly more common in large XLMR families with many affected males than in small families or sporadic male patients. Moreover, the

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existence of such modifiers would fit the observation that the male excess is highest in mild forms of MR.¹² So far, however, there is no empirical evidence for the existence of sequence variants on the X-chromosome that act as modifiers of intelligence.

MiRNAs are interesting candidates for harbouring such variants because individual miRNAs can regulate the expression of several different target genes, whereas a single target gene may be under the control of several different miRNAs.¹³ Therefore, individual mutations in miRNAs may not be sufficient to cause disease but still able to act as genetic modifiers.

In order to investigate the involvement of miRNA mutations in XLMR, either as modifiers of intelligence or as sole causes of XLMR, we have examined all known, brain-expressed X-chromosomal miRNAs in a cohort of 464 mostly small families with nonsyndromic XLMR. This led to the identification of four nucleotide changes, located in three different miRNA genes.

Materials and methods

The sequences of pre-miRNA and the corresponding genome regions were obtained from the microRNA registry (<http://www.sanger.ac.uk/software/Rfam/>). Thirteen brain-expressed X-chromosomal miRNAs (see Table 1) were selected based on the expression data published by Bentwich *et al.*¹⁴ Genome regions covering the 13 pre-miRNAs were screened in a panel of 464 XLMR patients collected through the European XLMR consortium (<http://www.euromrx.com/>). Of the 464 patients, 33 belong to families with defined linkage intervals on the X chromosome whereas the remaining 431 males are from small families with possible XLMR. For variants identified in patients, a panel of 183 male controls was screened.

After PCR amplification (see Table 1 for primer sequences; reaction conditions are available upon request), patient PCR products were pairwise pooled for mutation

screening by denaturing high-performance liquid chromatography (DHPLC with the WAVE[®], Transgenomic), using 2–4 different temperatures as described previously.¹⁵ Mutations were verified by Sanger sequencing.

Results and discussion

MiRNAs are initially transcribed as long RNA precursor (pri-miRNAs) and then processed in the nucleus by the endonuclease Drosha to form approximately 60-nucleotide hairpin structures (pre-miRNAs). After export into the cytoplasm Dicer, a second endonuclease, catalyses the maturation of pre-miRNAs into approximately 22 nt RNA duplexes.¹⁶ Based on the assumption that mutations, either in the mature miRNA and/or the hairpin pre-miRNA are most likely to affect the processing and/or target selection of miRNAs, the pre-miRNA sequences of all brain-expressed miRNAs were screened. Of the 13 screened pre-miRNAs, four nucleotide changes in three different pre-miRNA hairpins (Figure 1) were identified in four unrelated patients. Each of the four changes occurred only once in our panel of 464 patients. None of the changes is listed as a known SNP in the relevant databases, but one of them (the variation in mir-223) was subsequently found in one out of 183 controls. The two variations found in mir-222 co-segregated with the disease in the respective families, whereas the change in mir-363 is absent in the proband's affected brother. All four changes are located outside the mature miRNA sequences.

The variations found in mir-363 and the C to U change in mir-222 are located in the stem regions of the respective pre-miRNA hairpins. Whereas the original sequences of these sites lead to C to G pairing, the two changes result in U to G pairing. Therefore, these variations do not alter the integrity of the stem and could be functionally neutral. Similarly, the A to U change in mir-222 does not change the mismatch status of the position and thus may not affect the stem stability either. However, it has been reported that during miRNA maturation, Drosha cuts

Table 1 miRNA coordinates and PCR amplicons

ID	Start ^a	End ^a	Forward primer	Reverse primer	Amplicon (bp)
mir-222	45362675	45362784	CCAGAAGGCAAAGGATCAC	TGCCCAATAATCTCTCAGG	166
mir-221	45361839	45361948	TTCTTGGTCTTCTCTG	CCCAGCATTTCTGACTGTTG	344
mir-362	49476608	49476672	AAACACACAAAAAGGGCAGG	TGAAAGAAGGGGACAGGTTG	167
lef-7f-2	53467174	53467256	TTCTCATGAAGTGCACACC	AGGCTGAAGATGGACACTGG	306
mir-223	65021733	65021842	TCCCTATTCTGGTGCTTGG	TGAGAACATTGGTGTCTGGT	327
mir-363	133028928	133029002	TGTAGCGCTGATCAGGAATG	TTCATGCGTATCTCCAGCAC	304
mir-19b-2	133029221	133029316	TGCTGGAGATAACGCGATGAAG	AACAGATGGTGGGGACTGTG	315
mir-92-2	133029088	133029162	TGTAGCGCTGATCAGGAATG	TTCATGCGTATCTCCAGCAC	304
mir-106a	133029748	133029828	AGGGGCATTAGGGCAGTAG	TGAGGACGGAAAAGAAGAGC	286
mir-504	137475392	137475474	ATTTCATGAAGCATTGGGG	TACAGGGGACGAACTGATCC	342
mir-105-1	151231259	151231339	AACAAATGGCTTGGTCCAG	TGGATGTCAGCTTCTGTTGC	155
mir-105-2	151233452	151233532	GCTTGTCCAGAACGAGATG	TGGTATCAGCTCATGTTGAG	148
mir-505	138731827	138731910	ATGAGCAAATGAATGGGAGC	CTTGAAGCAGAACAGAGGCC	188

^aChromosome position of miRNA in bp, based on the NCBI genome build 35.

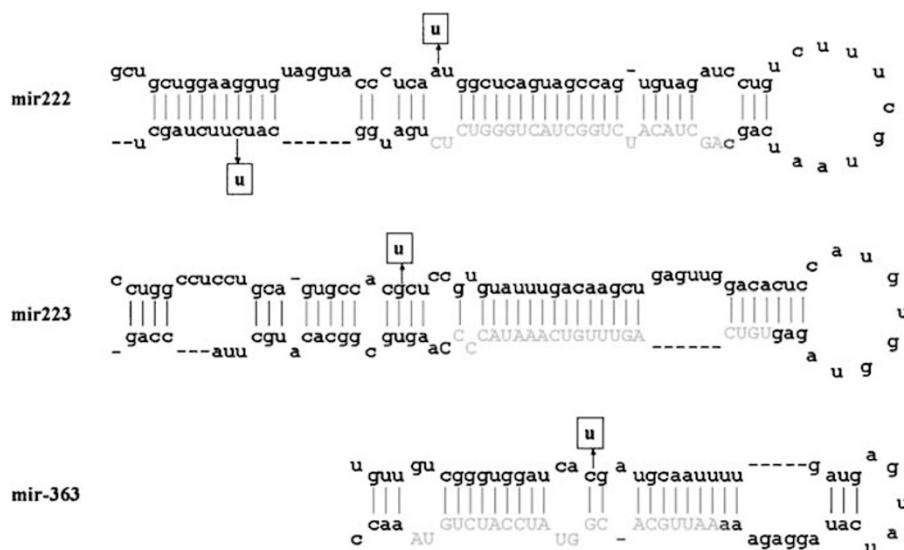


Figure 1 Variations in pre-miRNA sequences: pre-miRNA hairpins are shown, with gray capital letters indicating the respective mature sequence. Boxed letters represent the observed sequence changes.

pre-miRNAs at a distance of ~22 nt from the terminal loop-stem junction.¹⁷ If this is also the case for mir-222, the A to U change would be quite close to the Drosha cleavage site and consequently, it might be able to influence the processing of its RNA product. However, when using ectopically expressed mir-222 constructs a detectable influence on Drosha processing could not be observed (David Bartel and Jinkuk Kim, unpublished data).

In the present study, mutation screening was confined to the pre-miRNA hairpins. It cannot be excluded, that mutations outside the pre-miRNA might also interfere with miRNA processing by affecting the pri-miRNA structure and variations in promoter regions may influence miRNA expression. The analysis of these regions, however, requires the clarification of pri-miRNA structure and unambiguous definition of miRNA promoters, of which little is known so far.

This study represents the first large-scale mutation screening of miRNAs in patients with mental retardation. The rarity of the nucleotide changes found in the investigated pre-miRNA hairpins and the fact that most of them appear to be functionally neutral may reflect strong selection acting on these regions and thus indicate the general importance of the miRNA system. Although so far, our results have failed to show an obvious role of miRNAs in the aetiology of mental retardation, our investigations do not rule out this possibility. Dysfunction of genes that are important for brain structure and function may also result from genetic variation of miRNA target sequences, for example, in 3' untranslated regions. The analysis of such target sequences in large cohorts of families may shed more light on the role of miRNAs in the pathogenesis of nonsyndromic XLMR.

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References

- 1 Reinhart BJ, Slack FJ, Basson M et al: The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* 2000; **403**: 901–906.
- 2 Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM: Bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in *Drosophila*. *Cell* 2003; **113**: 25–36.
- 3 Chen CZ, Li L, Lodish HF, Bartel DP: MicroRNAs modulate hematopoietic lineage differentiation. *Science* 2004; **303**: 83–86.
- 4 Poy MN, Eliasson L, Krutzfeldt J et al: A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* 2004; **432**: 226–230.
- 5 Giraldez AJ, Cinalli RM, Glasner ME et al: MicroRNAs regulate brain morphogenesis in zebrafish. *Science* 2005; **308**: 833–838.
- 6 Stevenson RE, Schwartz CE, Schroer RJ: *X-linked Mental Retardation*. Oxford: Oxford University Press, 2000.
- 7 Ropers HH, Hamel BC: X-linked mental retardation. *Nat Rev Genet* 2005; **6**: 46–57.
- 8 Fishburn J, Turner G, Daniel A, Brookwell R: The diagnosis and frequency of X-linked conditions in a cohort of moderately retarded males with affected brothers. *Am J Med Genet* 1983; **14**: 713–724.
- 9 Biancalana V, Beldjord C, Taillandier A et al: Five years of molecular diagnosis of Fragile X syndrome (1997–2001): a collaborative study reporting 95% of the activity in France. *Am J Med Genet A* 2004; **129**: 218–224.
- 10 Gronskov K, Hjalgrim H, Nielsen IM, Brondum-Nielsen K: Screening of the ARX gene in 682 retarded males. *Eur J Hum Genet* 2004; **12**: 701–705.
- 11 Mandel JL, Chelly J: Monogenic X-linked mental retardation: is it as frequent as currently estimated? The paradox of the ARX

- (Aristaless X) mutations. *Eur J Hum Genet* 2004; **12**: 689–693.
- 12 Leonard H, Wen X: The epidemiology of mental retardation: challenges and opportunities in the new millennium. *Ment Retard Dev Disabil Res Rev* 2002; **8**: 117–134.
- 13 Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004; **116**: 281–297.
- 14 Bentwich I, Avniel A, Karov Y *et al*: Identification of hundreds of conserved and nonconserved human microRNAs. *Nat Genet* 2005; **37**: 766–770.
- 15 Jensen LR, Lenzner S, Moser B *et al*: X-linked mental retardation: a comprehensive molecular screen of 47 candidate genes from a 7.4 Mb interval in Xp11. *Eur J Hum Genet*, advance online publication, 13 September 2006.
- 16 Lee Y, Jeon K, Lee JT, Kim S, Kim VN: MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J* 2002; **21**: 4663–4670.
- 17 Han J, Lee Y, Yeom KH *et al*: Molecular basis for the recognition of primary microRNAs by the Drosha–DGCR8 complex. *Cell* 2006; **125**: 887–901.