

RESEARCH

Screening for mutations in selected miRNA genes in hypogonadotropic hypogonadism patients

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Abstract

In approximately half of congenital hypogonadotropic hypogonadism (cHH) patients, the genetic cause remains unidentified. Since the lack of certain miRNAs in animal models has led to cHH, we sequenced human miRNAs predicted to regulate cHH-related genes (*MIR7-3*, *MIR141*, *MIR429* and *MIR200A-C*) in 24 cHH patients with Sanger sequencing. A heterozygous variant in *MIR200A* (rs202051309; general population frequency of 0.02) was found in one patient. Our results suggest that mutations in the studied miRNAs are unlikely causes of cHH. However, the complex interplay between miRNAs and their target genes in these diseases requires further investigations.

Key Words

- ▶ microRNA
- ▶ mutation
- ▶ hypogonadotropic hypogonadism
- ▶ Kallmann syndrome

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Introduction

Congenital hypogonadotropic hypogonadism (cHH) is a rare genetic disease that prevents pubertal development and causes infertility due to deficient secretion or action of gonadotropin-releasing hormone (GnRH) (1). Congenital hypogonadotropic hypogonadism is called normosmic (ncHH) if patients have normal sense of smell, whereas Kallmann syndrome (KS) is a form of the same disease where patients have absent or deficient smell (2). In the case of normosmic cHH, abnormal GnRH function results from mutations affecting GnRH signaling, whereas in the case of KS, development of the olfactory system along with the development and/or migration of the GnRH neurons are disrupted (1, 3). These diseases have great phenotypic and genetic heterogeneity, as to date over 30 genes underlying ncHH and KS have been identified (1). Several ncHH and KS disease genes are yet to be discovered, since currently known genes account for only half of all cases (1).

miRNAs are small (~22 nt long) non-coding RNAs that suppress gene expression by binding with 3'UTRs of their target mRNAs. Binding of a miRNA with its target

mRNA induces the mRNA's translational repression or decay (4). miRNAs from individual gene families typically target hundreds of mRNAs, and over 60% of human protein-coding genes are miRNA targets (5). The significance of miRNAs in the hypothalamus–pituitary–gonadal system regulation has been shown in several animal models. Garaffo *et al.* showed that miR-200 (named miR-8 in miRBase) and miR-9 gene families are required for early GnRH neuron genesis and migration and that lack of these miRNAs lead to KS phenotypes in zebrafish (6, 7). In turn, Ahmed *et al.* demonstrated that homozygous knockout mice lacking miR7a-2, a miR-7 family member and precursor, recapitulated the phenotype of normosmic cHH (8). Therefore, we asked if some miRNA genes (or their closest human equivalents), which are known to regulate the hypothalamus–pituitary–gonadal system in animals, could be mutated in ncHH or KS patients without genetic diagnoses. Members of the miR-200 (miR-8) gene family, *MIR141*, *MIR429* and *MIR200A-C*, were selected for screening based on previous evidence (6, 7) and

MIR7-3 based on literature (9) and bioinformatic analyses by using miRWalk 3.0 (10, 11) and BLAT (12) and BLASTN (13) tools in Ensembl (14).

Subjects and methods

We studied a set of 19 KS and 5 normosmic cHH patients without previously found mutations in known cHH-causing genes (26, 27). Informed consent was obtained from all patients, and in the case of minor/children, a parent or guardian gave the consent. The study was approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa, and it was conducted in accordance with the Declaration of Helsinki.

The RNA-coding exons and exon-untranslated region boundaries of *MIR141* (ENSG00000207708, ENST00000384975.1), *MIR429* (ENSG00000198976, ENST00000362106.1), *MIR200A* (ENSG00000207607, ENST00000384875.3), *MIR200B* (ENSG00000207730, ENST00000384997.3) and *MIR200C* (ENSG00000207713, ENST00000384980.3) were amplified by means of PCR from the genomic DNA of the Kallmann patients and the equivalent regions of *MIR7-3* (ENSG00000207630, ENST00000384898.1) from the genomic DNA of the ncHH patients. The PCR conditions and primers are available upon request. The PCR products were purified with ExoProStar treatment (GE Healthcare Life Sciences) and sequenced from both directions with the ABI BigDye Terminator Cycle Sequencing Kit (v3.1) and ABI Prism 3730xl DNA Analyzer automated sequencer (Applied Biosystems). The DNA sequences were aligned and read with Sequencher 4.9 software (Gene Codes Corporation, Ann Arbor, MI, USA). Allele frequency of the identified variant was validated from the Genome Aggregation Database (gnomAD) (<http://gnomad.broadinstitute.org/>) (28). gnomAD contains WGS and exome data of 141,456 individuals including 12,562 Finnish samples.

The mouse gene *Mir7-2* was aligned against the human reference genome (GRCh38) with the BLAT (12) and BLASTN (13) tools in Ensembl database (14). The BLAT search was run with 'Genomic sequence' and the BLASTN search with 'Ensembl Non-coding RNA genes' as DNA databases. In both alignment types, other settings were default and the mouse reference genome (CL57BL6) was applied as a control genome. BLAT found hits in two genes, *MIR7-3* and *MIR7-3HG*, with score 46 (probability 3.6e-05). BLASTN found hits in several genes, among which *MIR3529* and *MIR7-2* had the highest score (52, probability 5e-07) and *MIR7-3* and *MIR7-1* the second-highest score (48.1, probability 9e-06).

miRWalk 3.0 (<http://mirwalk.umm.uni-heidelberg.de/>) search with default settings was applied to find the predicted target genes of hsa-miR-7-5p (7).

Results

To find out if mutations in miRNA genes, which are predicted to regulate the hypothalamus–pituitary–gonadal system, could underlie KS/ncHH, our 19 KS patients were screened for mutations in *MIR141*, *MIR429* and *MIR200A-C*, and 5 ncHH patients for mutations in *MIR7-3*. We amplified and Sanger-sequenced the coding exons and exon–intron boundaries of the selected genes from the patient genomic DNA. A heterozygous variant, c.42C>T, rs202051309, with a frequency of 0.01996 in the Finnish population in gnomAD, was found in *MIR200A* in one KS patient. There were no mutations in other investigated miRNA genes.

Discussion

Mutations in miRNA genes may alter the target specificity and processing of miRNAs and cause disease (4, 15). For example, a mutation in the *MIR96* gene has been shown to alter the miR-96 biogenesis and, consequently, cause autosomal dominant deafness in an Italian family (16). A SNP in *MIR140* is known to alter the miR-140 precursor processing and be associated with familial isolated cleft palate (17), whereas a mutation in *MIR184* underlies familial severe keratoconus combined with early-onset cataract (18). To the best of our knowledge, however, miRNA genes have not been investigated in patients with cHH to date.

Members of the miR-200 (miR-8) family are expressed in the mouse and zebrafish olfactory tissues (19, 20) and are required for the olfactory progenitor cell differentiation in mice (20). Moreover, miR-200 miRNAs are expressed in the GnRH neurons and pituitary, and they play a role in reproduction and fertility in mice (21, 22). Lack of miR-200 members recapitulated KS phenotypes in zebrafish (6). As previous studies strongly indicate their role in the olfactory system and GnRH neuron development, genes of the miR-200 (miR-8) family, *MIR141*, *MIR429*, *MIR200A*, *MIR200B* and *MIR200C*, were chosen for screening in our KS patients. In the current study, we identified one variant (c.42C>T, rs202051309) in *MIR200A* in one of our KS patients. As its general population frequency was relatively high (0.01996), we concluded that it is unlikely to cause disease. However, our patient cohort was limited in size and we cannot fully exclude the possible effect of

this variant on target specificity or miRNA processing. Indeed, if one assumes that mutations in the mir-200 family genes selected for the current study caused 10% of KS, we would have had an 86% chance to detect at least one such a mutation among our 19 KS patients.

Based on the results by Ahmed *et al.* (8), literature (9) and our bioinformatic analyses, we chose *MIR7-3* for screening of our normosmic cHH patients. In brief, all human miR-7-encoding genes (*MIR7-1*, *MIR7-2* and *MIR7-3*) produce primary miRNAs that are subsequently processed into pre-miRNAs and finally into the same mature miR-7 (4, 9). Most of the miR-7 expression in the human pituitary is presumably attributed to *MIR7-3* that is located in an intron of the pituitary-specific gene *PGSF1* (pituitary gland specific factor 1, also known as *MIR7-3HG*, *MIR7-3* host gene) (23, 24, 25). In addition, the predicted target genes of the mature human miR-7, hsa-miR-7-5p, include several of the murine miR-7 predicted target genes, such as *Glg1*, *Ptgfrn*, *Sema4c* and *Chd3* (8) and currently known nHH/KS genes such as *GNRHR*, *FGFR1*, *SEMA7A* and *PROK2* (see Subjects and methods; (1)). However, we found no mutations in *MIR7-3*, which implies it might rarely be mutated in nHH, suggesting that the miRNA it encodes has no implications in the human cHH.

In conclusion, this study is the first on miRNAs in cHH. Based on our results, mutations in the examined miRNAs seem to be a rare cause of cHH. We acknowledge that our approach is limited, as we selected specific RNA genes with implicated significance in animal experiments. An unbiased human tissue RNA expression analysis might imply that the most central human and animal miRNAs differ in the hypothalamus–pituitary–gonadal axis. Thus, we cannot exclude the possibility that the examined, or other miRNAs, might contribute to the development and function of the hypothalamus–pituitary–gonadal axis in humans.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

T R conceived and designed the research, A I and J K performed the experiments and all authors participated in analyzing the results and writing the manuscript.

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