

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Bouligand J, Ghervan C, Tello JA, et al. Isolated familial hypogonadotropic hypogonadism and a *GNRH1* mutation. *N Engl J Med* 2009;360:2742-8. DOI: 10.1056/NEJMoa0900136.

Supplementary Appendix

Part 1 - GNRH1 and GNRH2 sequencing

Coding-exons 1, 2 and 3 and intron-exon junctions of *GNRH1* and coding-exons 2, 3 and 4 and intron-exon junctions of *GNRH2* were amplified by polymerase chain reaction (PCR) using genomic DNA as a template. PCR was performed in a final volume of 20 μ L containing 50 ng of genomic DNA, 10 pmol of sense and antisense oligonucleotides, 200 μ M each dNTP, 1.5 mM MgCl₂, PCR buffer (pH 8.3) and 0.2 U of Taq Polymerase (Yellow Star[®], Eurogentec[™], Angers, France). Following denaturation for 4 minutes at 94°C, 30 cycles of amplification were carried out in a thermocycler. Each cycle consisted of an initial 30-second denaturation at 94°C, 1-minute annealing at 58°C and 1-minute elongation at 72°C. The amplification cycles were followed by a final elongation step of 8-minutes at 72°C. The PCR products were sequenced in both directions by using sense or antisense primers and the Big Dye terminator kit v1.1 (Applied Biosystems[™], Foster City, CA). The same primers (Table 1S) were used for PCR and sequencing. The conditions employed were as recommended by the manufacturer, with an initial 3-minute denaturation step at 96°C and 30 cycles with a 10-second denaturation at 96°C, 5-second annealing at 50°C and 4-minute elongation at 60°C. Linear amplification products were then separated in an automated capillary sequencer (ABI PRISM 3130xl Genetic Analyzer, Applied Biosystems[™], Foster City, CA). Electropherogram-derived sequences were compared to NCBI reference

sequences for either *GNRH1* (GenBank accession numbers: contig NC_000008.9 and transcripts NM_000825.3 and NM_001083111.1) or *GNRH2* (GenBank accession numbers: contig NC_000020.9 and transcript NM_001501.1) using Seqscape[®] software (Applied Biosystems[™], Foster City, CA)

Table 1S: Primers for *GNRH1* and *GNRH2* PCR amplification and sequencing

Name	Amplicon size	Sense primers	Antisense primers
GNRH1-exon1	383 bp	CCATCTTCTGCAGGGTTAGTG	GCCTTATCTCACCTGGAGCA
GNRH1-exon2	230 bp	GGTGGAAATGGAAAACACCA	ATCCCCAAAGGCTTATCCAC
GNRH1-exon3	384 bp	CAAACAGACCAATTCCTTCCA	TCCCTTTGGTGGGTTTACAG
GNRH2-ex2/3	575 bp	AGTGGCCCTGGAGGAAGTAG	ACCCAACCCTAAGCCACAAT
GNRH2-exon4	176 bp	AGGGGAGAGAACCAGGAAGA	AGGGGACGAAGGACAGAACT

Legend to table 1S :

The sense and antisense primers were designed with the online version 0.4 of Primer 3. All primers were blasted to check their selectivity on the NCBI website, using the Human Genomic Plus Transcript database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences in bold indicate exonic primers, while the others are intronic.

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Part 2 - Variable Number Tandem Repeat (VNTRs) Analysis

New and commercial VNTRs (Linkage mapping set, Applied Biosystems™) were amplified by PCR using genomic DNA as template. PCR was performed in a final volume of 20 µL containing 30 ng of genomic DNA, 10 pmol of sense and antisense oligonucleotides, 200 µM each dNTP, 1.5 mM MgCl₂, PCR buffer and 0.2 U of Taq Polymerase (Yellow Star®, Eurogentec™, Seraing, Belgium). Sense primers (new primers for VNTRs) were labeled by the fluorochrome FAM in 5' (Table 2S). Following denaturation for 3 min at 94°C, 30 cycles of amplification were carried out in a thermocycler. Each cycle consisted of initial 30-s denaturation at 94°C, 30-s annealing at 56°C and 30-s elongation at 72°C. Followed by a final elongation step of 10-min at 72°C. The fluorescent PCR products were analyzed in an automated capillary sequencer (ABI PRISM 3130xl Genetic Analyzer, Applied Biosystems™). Amplicons sizes were determined by comparison with size markers with Genscan® analysis software (Applied Biosystems™).

Table 2S: Primers for VNTRs analysis around GNRH1 locus (-14 Mb to +32 Mb).

Name (localisation)	Type	Sense primer (New VNTRs with 5'FAM)	Antisense Primer	Position (Kbp)
D8S285 (+32 Mb)	CA repeat	D8S285F (ABI panel 12)	D8S285R (ABI panel 12)	8p12 – 57.230 Kbp
D8S505 (+9 Mb)	CA repeat	D8S505F (ABI panel 12)	D8S505R (ABI panel 12)	8p12 – 34.751 Kbp
VNTR8-895 (+2 Mb)	CA repeat	CCAGCATTTTACAGGGCTAT	TCAGTTACCCCAAGGGAAAT	8p21 – 26.826 Kbp
D8S1771 (+0.2 Mb)	TG repeat	D8S1771F (ABI panel 12)	D8S1771R (ABI panel 12)	8p21 – 25.497 Kbp
VNTR8-966 (+43Kb)	TG repeat	CTGCAGTGTTGTCCTCCAAA	GAAGACCCCAAGGGCTTACTC	8p21 – 25.382 Kbp
VNTR8-665 (+1Kb)	CA repeat	TCCAATTGGTTTGTGTTTGC	ACCTCCCAGCCTACATCCTT	8p21 – 25.340 Kbp
VNTR8-197 (-22Kb)	TG repeat	TGTGCCAGAGGTTGTATGGA	GTGCAAAGCTCCTGCTCTTC	8p21 – 25.310 Kbp
VNTR8-963 (-2 Mb)	TG repeat	AGTGCAGTGGTGTGAGCTTG	CTCCCTTCTTCCGAATGTT	8p21 – 23.329 Kbp
D8S258 (-5 Mb)	TG repeat	D8S258F (ABI panel 11)	D8S258R (ABI panel 11)	8p21p22 – 20.412 Kbp
D8S549 (-10 Mb)	CA repeat	D8S549F (ABI panel 11)	D8S549R (ABI panel 11)	8p22 – 15.694 Kbp
D8S550 (-14 Mb)	TG repeat	D8S550F (ABI panel 12)	D8S550R (ABI panel 12)	8p23 – 10.919 Kbp

Legend to table 2S :

New and commercial VNTRs (Applied Biosystems™) are localized from -14Mb to +32 Mb around the *GNRH1* locus (25.339 Kbp to 25.332 Kbp, negative strand) on the short arm of the chromosome 8 (8p21). New VNTRs primers were designed according to the microsatellite database (<http://www.microsatellites.org/about.htm>). The sense and antisense primers were designed with the online version 0.4 of Primer 3. All primers were blasted to check their selectivity on the NCBI website, using the Human Genomic Plus Transcript database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Their precise chromosomal

localization were verified online by electronic-PCR (<http://www.ncbi.nlm.nih.gov/sutils/e-pcr/reverse.cgi>).

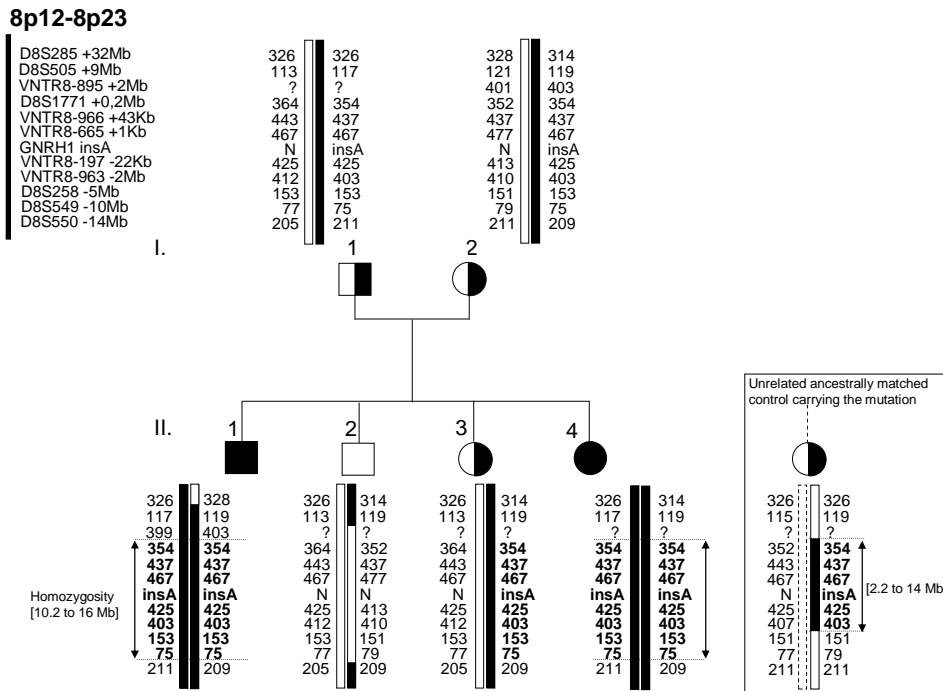


Figure 1S – Pedigree and Haplotype Analysis

Variable number tandem repeats (VNTRs: CA or TG repeats) from -14 Mb to +32 Mb surrounding the *GNRH1* gene were analyzed for all the family members and for an unrelated ancestrally matched control carrying the heterozygous mutation c.18-19 ins A of *GNRH1*. The amplicon sizes (bp) determined for each VNTR were used to construct the haplotypes and are reported for each person in the pedigree. The homozygous segment range is indicated by a double arrow and bold characters in the two affected family members (II.1 and II.4). A double arrow for the unrelated ancestrally matched heterozygous control indicates the common haplotype encompassing the mutation.

Supplementary Appendix

Part 3 - In vitro Analysis of the *GNRH1* mutation

Primers used for in vitro mutagenesis:

5'-ttctaaggcacatgaatgcac-3'

5'-tgaagccaattcaaaaaactcctagctgg-3'.

AtT-20 cells were grown in 6 cm cell culture plates (Corning Inc., USA) and transfected with the indicated expression constructs using Fugene 6 (Roche Diagnostics GmbH, Germany). They were maintained in OPTI-MEM serum reduced media (Invitrogen, USA) and the conditioned media was

collected 48 hours post-transfection then snap frozen on dry ice. GnRH was measured in conditioned media by radioimmunoassays.