

# Supplementary Appendix

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

Supplement to: Seminara SB, Messager S, Chatzidaki EE, et al. The GPR54 Gene as a Regulator of Puberty. *N Engl J Med* 2003;349:1614-27.

**MUTATION ANALYSIS**

Exons 1, 2, 4, and 5 were amplified with the use of the GC-rich PCR system with its accompanying PCR program (Roche Diagnostics). Sequencing was performed with the use of an ABI 377 automated sequencer (Applied Biosystems). The primers used for genomic screening were as follows:

GPR54-e1F: 5'-GCTGGGTGAATAGAGGGC-3', GPR54-e1R: 5'-GGAGTTTGGCACC-TCTAGC-3'; GPR54-e2F: 5'-CCATCCTGCTGGTCACTCG-3', GPR54-e2R: 5'-CACTG-CGGAGCGCACTCC-3'; GPR54-e3F: 5'-GCCTGAGTGTTCGCACACG-3', GPR54-e3R: 5'-GCGCCCATTTCCAGATGC-3'; GPR54-e4F: 5'-GCATCTGGAAAATGGGCGC-3', GPR54-e4R: 5'-GGAAGGCGTAGAGCAGCG-3'; GPR54-e5F: 5'-GGAGGACAGCAAG-GCTGG-3', GPR54-e5R: 5'-AAACTGCACCGAACGTCACA-3'.

DNA extracted from 48 of 50 samples from persons from the Middle East and 50 samples from black persons in North America were derived from Coriell Cell Repositories (Camden, N.J.).

Control panel for use with the samples from black persons (HD50AA):

NA17101, NA17102, NA17103, NA17104, NA17105, NA17106, NA17107, NA17108, NA17109, NA17110, NA17111, NA17112, NA17113, NA17114, NA17115, NA17116, NA17117, NA17118, NA17119, NA17120, NA17121, NA17122, NA17123, NA17124, NA17125, NA17126, NA17127, NA17128, NA17129, NA17130, NA17131, NA17132, NA17133, NA17134, NA17135, NA17136, NA17137, NA17138, NA17139, NA17140, NA17141, NA17142, NA17143, NA17144, NA17145, NA17146, NA17147, NA17148, NA17149, NA17150.

Control panel for use with the samples from persons in the Middle East, version 1 (HD05) and version 2 (HD27):

NA17041, NA17042, NA17043, NA17044, NA17045, NA17046, NA17047, NA17048, NA17049, NA17050, NA17331, NA17332, NA17333, NA17334, NA17335, NA17336, NA17337, NA17338, NA17339, NA17340; Individual Middle-Eastern control samples: NA02016, GM13350, NA11811, GM13439, NA13395, NA11521, NA11522, NA11523, NA11524, NA11525, NA13989, GM00710, GM16180, GM06417, GM05880, GM01607, NA03388, GM02359, NA08925, GM00695, NA08100, NA07711, GM02990, NA02994, GM02992, GM02993, GM02995, GM02997.

**ALLELE-SPECIFIC CLONING**

The primers used for allele-specific cloning were as follows:

GPR54asF: 5'-CGAGGGGATGAGGCTGAGC-3', GPR54asR: 5'-CAAAC TTCACAAC-GAAACTGG-3'.

**RT-PCR**

Total RNA from lymphoblastoid cell lines was extracted with the use of the RNeasy Midi/Maxi kit (Qiagen). The primers used for RT-PCR were as follows:

GPR54-extAF: 5'-CTCTGGACCCTGCGGACC-3', GPR54-extAR: 5'-CAGGTGGCGCA-GCATGGC-3'; GPR54-intA1F: 5'-AGCCCCTTCCTGAGTTCCA-3', GPR54-intA1R: 5'-CGGTCAGAGTGGCACACG-3', GPR54-intA2F: 5'-CGTGACCTTCCTCCTGTGC-3', GPR54-intA2R: 5'-GGTGACAGGCGGTGCAGG-3'; GPR54-extBF: 5'-CGACTTCATG-TGCAAGTTCG-3', GPR54-extBR: 5'-AAACTGCACCGAACGTCACA-3'; GPR54-intB1F: 5'-GCCATGAGTGTGGACCGC-3', GPR54-intB1R: 5'-CCAGGAACAGCTGGATGG-3', GPR54-intB2F: 5'-CGCCTACTGCAGTGAGGC-3', GPR54-intB2R: 5'-CAGAAGAATA-GCCGCTGTCC-3'.

#### **GENERATION OF MUTANT CONSTRUCTS**

All mutants were created with the use of the QuikChange site-directed mutagenesis kit (Stratagene). The primers for site-directed mutagenesis were as follows (the boldface nucleotides represent the incorporated mutations):

1) GPR54-LtoS-F: 5'-CGGTGTTCCCGTGC GCGCCCTGCAC-3', GPR54-LtoS-R: 5'-GTGCAGGGCGCGC GACGGGAACACCG-3'; 2) GPR54-RtoX-F: 5'-CTGGGCTCGCACTTCTGACAGGCCTTCCGCC-3', GPR54-RtoX-R: 5'-GGCGGAAGGCTGTCAGAA GTGCGAGCCCAG-3'; 3) GPR54-XtoR-F: 5'-GACAACGCCCTCTCAGAGCGGACC CGGTGG-3', GPR54-XtoR-R: 5'-CCACCGGGTCCGCTCTGAGAGGGGCGTTGTC-3'; 4) GPR54-PostPolyA-F: 5'-AAAAAAAAAAAAAAAAAGTGAGCCGCTCTAGAGTATCCCTCGAGG-3', GPR54-PostPolyA-R: 5'-CCTCGAGGGATACTCTAGAGCGGCCTCACTT TTTTTTTTTTTT-3'.

#### **SELECTION OF LIGAND**

Kisspeptin-1 112–121 was synthesized by the Massachusetts General Hospital Peptide Synthesis–Protein Sequencing Core Laboratory for all the in vitro assays.

#### **INOSITOL PHOSPHATE ASSAYS**

COS-7 cells were transiently transfected with 1.5 µg of each GPR54 construct or empty vector per well, with the use of PolyFect Transfection Reagent (Qiagen).

#### **QUANTITATIVE RT-PCR**

Different primers and probes capable of amplifying the R331X and X399R alleles selectively were designed and synthesized (FAM [Biosource International]; VIC and TET [Applied Biosystems]): 1) GPR54total-F: 5'-TACATCCAGCAGGTCTCGGTG-3', GPR54total-R: 5'-ACGTACCAGCGGTCCACACT-3', GPR54total-Probe: 5'-CACGTGTGC-CACTCTGACCGCC-3' (TET labeled); 2) GPR54X399R-F: 5'-GCCCCCTCTCAGAGCGGAC-3', GPR54X399R-R: 5'-AAATACTAATAACAGAAGAATAGCCGCTG-3', GPR54X399R-Probe: 5'-CGGTGGGAATCCGAGCGGC-3' (VIC labeled); 3) GPR54R331X-F: 5'-GCCCCCTCTCTGAGCGGAC-3', GPR54R331X-R: 5'-AAATACTAATAACAGAAGAATAGCCGCTG-3', GPR54R331X-Probe: 5'-CGGTGGGAATCCGAGCGGC-3' (FAM labeled).

#### **INJECTION OF GONADOTROPIN-RELEASING HORMONE**

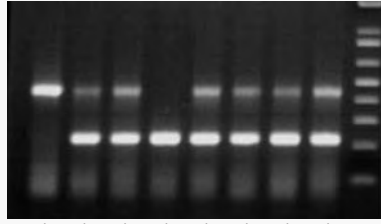
Gonadotropin-releasing hormone was provided by Calbiochem Behring.

#### **HORMONE ASSAYS IN MICE**

The immunoradiometric-assay measurements of luteinizing hormone in mice and the radioimmunoassay measurements of follicle-stimulating hormone in mice were performed by the Center for Research in Reproduction, Ligand Assay and Analysis Core Laboratory, University of Virginia. Testosterone radioimmunoassays were performed by the National Health Service Department of Clinical Biochemistry and Immunology of Addenbrooke's Hospital, Cambridge, United Kingdom. 17β-estradiol was measured with an ELISA kit (IBL).

#### **HISTOLOGY**

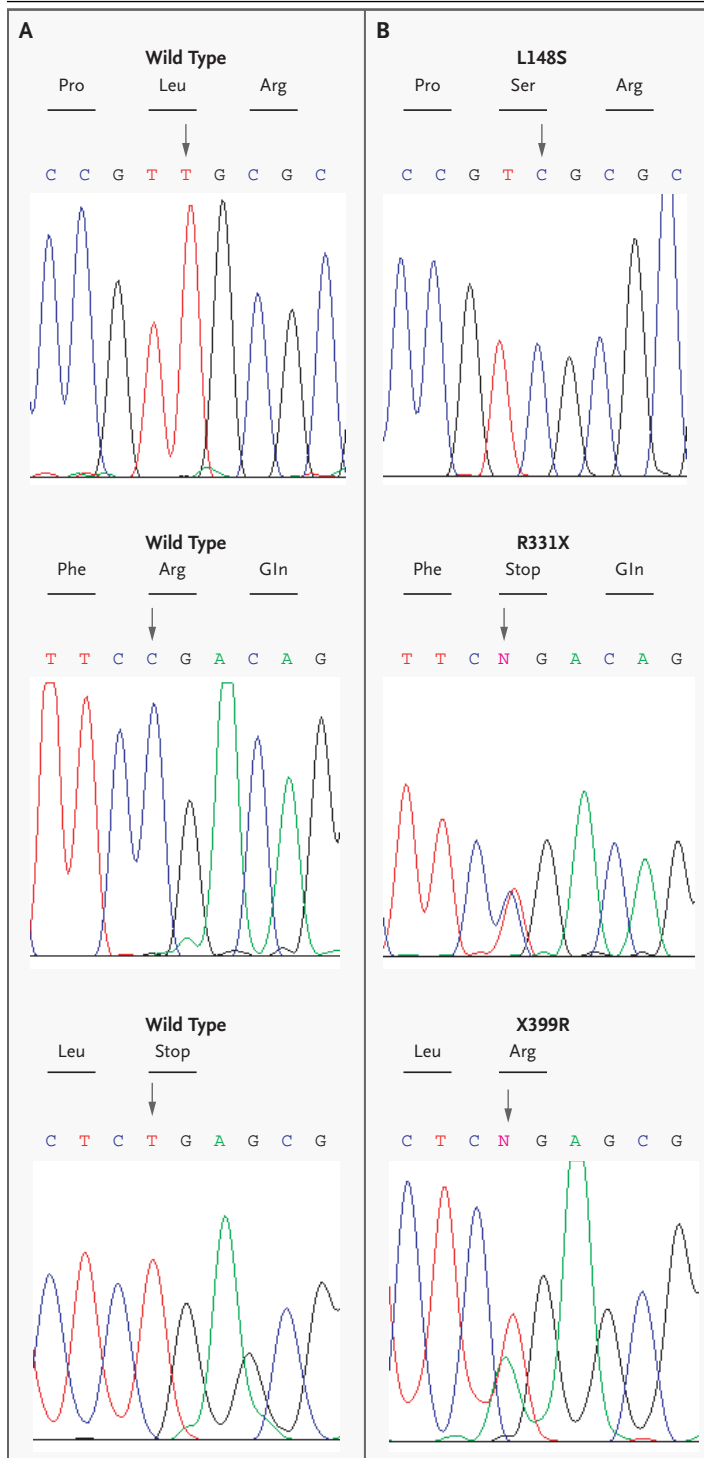
Sectioning of tissues was performed by the Histology Laboratory of the Veterinary School, University of Cambridge, United Kingdom. Carmine alum stain is composed of 1 g of carmine for every 2.5 g of aluminum potassium sulfate (both from Sigma-Aldrich).



-/- -/+ -/+ +/+ -/+ -/+ -/+ -/+

**Supplementary Appendix 2. Homozygous Deletions Detected on Multiplex PCR in Mice from Heterozygous Intercrosses.**

The presence of a wild-type band (lower band) or a mutant band (upper band) shows the genotype.



**Supplementary Appendix 3. Sequence Analysis of *GPR54* from an Affected Member of the Index Family (Panel A) and the Patient with the Compound Heterozygous Mutations (Panel B).**

The 443T>C in exon 3 substitutes a serine for the normal leucine; the 991C>T in exon 5 replaces an arginine with a premature stop codon; and the 1195T>A in exon 5 replaces the stop codon at residue 399 with an arginine.