Supplementary Material

Three novel missense mutations within the LHX4 gene are associated with variable pituitary hormone deficiencies. Roland W. Pfaeffle, Chad S. Hunter, Jesse J. Savage, Mario Duran-Prado, Rachel D. Mullen, Zachary P. Neeb, Urs Eiholzer, Volker Hesse, Nadine G. Haddad, Heike M. Stobbe, Werner F. Blum, Johannes F.W. Weigel, and Simon J. Rhodes

Mutation screening of *LHX4* gene fragments
Oligonucleotide sequences and amplified DNA fragments used for DHPLC-analysis:
(red: oligonucleotide sequence; uppercase: exon sequences)

Exon 1
forward primer: 5'-gcg gcc tgc ttg ggg ttt taa t-3'
reverse primer: 5'-tgc cct gtg acc gcc tct gc-3'
fragment length: 316 bp
WAVE fragment T's: 62.7°C/ 69°C

gcggcctgcttggggttttaattattattttgaaatttctgaatcgagctagagcgagagagcgagag
atctccgtagactgcgactcgctggctttcgctccgagATGATGCAGAGTGCGACTGTCCCCGCGGAA
GGGGCTGTCAAGGGGCTCCCGGAGATGCTAGGTGTGCCGATGCAACgtaagacacccccctttctcgc
tgatttaattctaacaagacagctagcagcctcagccgctgcggggcgggccggacgccgctcagggg
ccgggaggggctggcggccggggcgcagaggcggtcacagggca

Exon 2
forward primer 5'-gca ggg ctg tgt gga agg ctc-3'
reverse primer 5'-ctg gcc tac cct gct gca aac-3'
fragment length: 310 bp
WAVE fragment T's: 62.7°c

gcagggctgtgtggaaggctcacagtgcctctctctcctcttcctcacagAGATTCCCCAGTGCGCTG
GCTGCAACCAGCACATCCTGGACAAGTTCATCCTGAAGGTCCTGGACAGACACTGGCACAGCTCCTGC
CTCAAGTGTGCAGACTGCCAGATGCAGCTGGCGGACAGGTGCTTCTCCAGGGCTGGGAGCGTCTACTG
CAAGGAGGACTTCTTCAAgtaagtcagaacggtccgtctcgtggccctggcctgtctctgcctctccc
caagcagtgaggggaagtttgcagcagggtaggccag

Exon 3
forward primer: 5'-ttg ctc cct gtg tgc cct aat c-3'
reverse primer: 5'-ggt gca tga gta ggg ctc tga g-3'
fragment length: 366 bp
WAVE fragment T's: 63.6°C

ttgctccctgtgtgccctaatcctttcctgctgccctgacagGCGCTTCGGCACAAAATGCACGGCCT
GCCAGCAGGGTATCCCCCCAACCCAGGTGGTCCGCAAGGCCCAGGACTTTGTCTACCACCTGCACTGC
TTTGCTTGCATCATCTGCAACCGGCAGCTGGCCACGGGGGACGAATTCTACCTCATGGAGGACGGGCG
GCTGGTGTGCAAGGAAGACTACGAGACAGCCAAGCAGAACGgtaagcagcatggccccgcatggtccc
ctctccaggcctttgtttgggccacgccctctgcctgaggtgcccttctcatggcgtcccacctgccc
atccctcagagccctactcatgcacc

Exon 4
forward primer: 5'-gcc tcg cgc tgt cct gcc tac-3'
reverse primer: 5'-tga gcc caa cca ccc gtg ag-3'
fragment length: 377 bp
WAVE fragment T's: 62.6°C

gcctcgcgctgtcctgcctacagcaggcaggcttaggtgcagaaaggatggaagggagggtgtgggag
gaggcgcagctgctgcagataggccgaagccagtaagcagtggtttttccttgcagATGACTCAGAGG
CTGGAGCTAAGCGGCCCCGGACCACCATCACAGCCAAGCAGCTGGAGACATTAAAGAATGCATACAAG
AACTCCCCCAAGCCTGCCCGGCACGTGAGGGAGCAGCTGTCCTCAGAGACAGGCCTGGACATGAGGGT
CGTACAGgtgagatgccagcactcctgtgccctccggggatcccaggcccgggacaggggtggaaggt
atcctgagtgacatcagctcacgggtggttgggctca

Exon 5
forward primer: 5'-ggt gga cgc ccc ctg agt atg-3'
reverse primer: 5'-ggc ctc aag aaa gat ccc tcc-3'
fragment length: 307 bp
WAVE fragment T's: 62.6°C

ggtggacgccccctgagtatgtcccttgtgcttgtgtggcagGTTTGGTTTCAGAACAGAAGGGCCAA
AGAGAAACGCCTGAAGAAGGATGCAGGGCGGCACCGCTGGGGGCAGTTCTATAAGAGCGTCAAGAGGA
GCCGGGGCAGCAGCAAGCAGGAGAAGGAGAGCTCTGCAGAGGACTGTGGGGTTAGTGACAGTGAGCTG
AGCTTCCGAGgtgagcagggctggaggggccaggccgaggccttaggaaagtcccctgggaatctgta
atccctggcactcaggagggatctttcttgaggcc

Exon 6 (fragment 1)
forward primer: 5'-tgg cag ctg aca ata aat ctc c-3'
reverse primer: 5'-gcc caa att act gtc cac cgt-3'
fragment length: 277 bp
WAVE fragment T's: 62.6°C

tggcagctgacaataaatctccgttcttttgtccacagAGGATCAAATTCTCTCAGAACTTGGCCACA
CCAATAGGATTTATGGCAACGTGGGGGACGTTACAGGCGGACAGTTAATGAATGGGAGCTTCTCCATG
GACGGGACAGGACAATCCTATCAGGACTTGAGGGATGGGAGCCCCTATGGAATCCCCCAGTCTCCATC
CTCCATATCGTCCCTGCCATCCCACGCTCCTTTGCTCAATGGGCTGGATTACACGGTGGACAGTAATT
TGGGC

Exon 6 (fragment 2)
forward primer: 5'-tgc cat ccc acg ctc ctt tg-3'
reverse primer: 5'-gca ggt agg gtg ggg agg aga g-3'
fragment length: 240 bp
WAVE fragment T's: 62.6°C

TGCCATCCCACGCTCCTTTGCTCAATGGGCTGGATTACACGGTGGACAGTAATTTGGGCATCATTGCG
CATGCAGGGCAGGGAGTAAGCCAGACGCTGAGAGCCATGGCTGGGGGACCCACCTCTGACATCTCCAC
AGGAAGCAGTGTAGGCTATCCCGACTTTCCAACTAGCCCAGGCTCTTGGCTCGATGAAATGGATCATC
CTCCTTTTTAAacttctctcctccccaccctacctgc

PCR Amplification Conditions:
PCR reactions were performed on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with 100 ng of genomic DNA and 10 pmol of each forward and reverse oligonucleotide in a volume of 50 µl using 1,25 U of Qiagen Taq-Polymerase (Hilden, Germany), 10 pmol of NTPs and 5 µl of the reaction buffer supplied by the manufacturer.

PCR program	for exons 3 to 6-2		for exons 1 and 2	
Initial Denat.	95°	15 min	95°	15 min
35 cycles				
Denaturation	95°	1 min	95°	1 min
Annealing	59°	1 min	59°	1 min
Extension	72°	-	72°	1 min
Final Extension	72°	10 min	72°	10 min

DHPLC-Analysis:
All 7 fragments of the 6 coding exon with flanking intron sequences of the LHX4 gene were screened for mutations by dHPLC (Wave System®; Transgenomic Inc., Elancourt, France). For this purpose 10 µl of the patients amplified PCR product was mixed with the corresponding wildtype PCR product and then denatured at 95°C for 5 minutes and cooled via a temperature ramp of 1°C/minute to 37°C. Samples were kept cool until they were automatically inserted into a preheated reversed phase column (DNA-Sep; Transgenomic, San Jose, CA). DNA was eluted on a linear acetonitrile gradient consisting of buffer A (0.1 mol/L triethylammonium acetate; TEAA)/buffer B (0.1 mol/LTEAA, 25% acetonitrile). Gradient elution and melting temperature conditions were determined using Wave-Maker Navigator® software version 1.5.4 (Transgenomic). The analysis program calculates the melting temperature of the domains contained in the sequences of interest.
Experimental conditions (melting temperature and elution time shift) were optimized by studying alterations in the sample elution profiles. The optimized melting temperatures for each fragment used in our mutation screen are given above.