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Nasal Embryonic LHRH Factor (NELF) Mutations in Patients with Normosmic Hypogonadotropic Hypogonadism and Kallmann Syndrome

Ning Xu, PhD[1,2,*], **Hyung-Goo Kim, PhD**[1,*], **Balasubramanian Bhagavath, MD**[3], **Sung-Gyu Cho, MS**[4], **Jae Ho Lee, PhD**[5], **Kyungsoo Ha, MS**[6], **Irene Meliciani, MS**[7], **Wolfgang Wenzel, PhD**[7], **Robert H. Podolsky, PhD**[8], **Lynn P. Chorich, MS**[1], **Kathryn A. Stackhouse, BS**[1], **Anna M.H. Grove, BS**[1], **Lawrence N. Odom, MD**[1], **Metin Ozata, MD**[9], **David P. Bick, MD**[10], **Richard J. Sherins, MD**[11], **Soo-Hyun Kim, PhD**[12], **Richard S. Cameron, PhD**[5], and **Lawrence C. Layman, MD**[1]

[1]Section of Reproductive Endocrinology, Infertility, & Genetics, Department of Obstetrics & Gynecology; Developmental Neurobiology Program in the Institute of Molecular Medicine and Genetics; Neuroscience Program, The Medical College of Georgia, 1120 15th Street, Augusta, GA 30912

[2]Division of Endocrinology, Diabetes and Metabolism, Cedars-Sinai Medical Center, 8700 Beverly Boulevard, Becker Building, Los Angeles, CA 90048

[3]Reproductive Endocrinology and Infertility, Women and Infants Hospital, 101 Dudley Street, Providence, RI 02905

[4]Department of Cellular Biology and Anatomy, The Medical College of Georgia, Augusta, GA 30912

[5]Developmental Neurobiology Program, Institute of Molecular Medicine & Genetics, Medical College of Georgia, 1120 15th Street, Augusta, GA 30912

[6]MCG Cancer Center, Medical College of Georgia, 1120 15 street, Augusta, GA, 30912

[7]Karlsruhe Institute of Technology, Institute of Nanotechnology, PO Box 3640, 76021 Karlsruhe, Germany

[8]Center for Biotechnology and Genomic Medicine, Department of Medicine, The Medical College of Georgia, Augusta, GA 30912

[9]GATA Haydarpasa Training Hospital, Dept. of Endocrinology, Istanbul, 34660 Turkey

[10]Division of Medical Genetics, Departments of Pediatrics and Obstetrics & Gynecology, Medical College of Wisconsin, Milwaukee, WI 53226

[11]Director of Andrology, Columbia Fertility Associates, Washington, DC 20037

[12]Division of Basic Biomedical Sciences, St. George's Medical School, University of London, London, SW17 0RE, United Kingdom

Abstract

Correspondence should be addressed to Lawrence C. Layman, CA2041, IMMAG, Medical College of Georgia, 1120 15th Street, Augusta, GA 30912, USA. Phone: (706) 721-7591; Fax: (706) 721-0340; llayman@mcg.edu.
*Both N.X. and HG.K. contributed equally in this work

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Study Objective—To determine if mutations in *NELF*, a gene isolated from migratory GnRH neurons, cause normosmic idiopathic hypogonadotropic hypogonadism (IHH) and Kallmann syndrome (KS)

Design—Molecular analysis correlated with phenotype

Setting—Academic medical center

Patients—168 IHH/KS patients along with unrelated controls were studied for *NELF* mutations.

Intervention—*NELF* coding regions/splice junctions were subjected to PCR-based DNA sequencing, Eleven additional IHH/KS genes were sequenced in three patients with *NELF* mutations.

Main Outcome Measure—Mutations were confirmed by SIFT, RT-PCR, and western blot analysis.

Results—Three novel *NELF* mutations absent in 372-ethnically matched controls were identified in 3/168(1.8%) IHH/KS patients. One IHH patient had compound heterozygous *NELF* mutations (c.629-21C>G and c.629-23G>C); and he did not have mutations in 11 other known IHH/KS genes. Two unrelated KS patients had heterozygous *NELF* mutations and mutation in a second gene: *NELF/KAL1* (c.757G>A; p.Ala253Thr of *NELF* and c.488_490delGTT; p.Cys163del of *KAL1*) and *NELF/TACR3* (c. 1160-13C>T of *NELF* and c.824G>A; p.Trp275X of *TACR3*). *In vitro* evidence of these *NELF* mutations included reduced protein expression and splicing defects.

Conclusions—Our findings suggest that *NELF* is associated with normosmic IHH and KS, either singly or in combination with a mutation in another gene.

Keywords

Nasal embryonic LHRH factor; hypogonadotropic hypogonadism; Kallmann syndrome; gonadotropin-releasing hormone (GnRH); GnRH neuron migration

Introduction

Normal puberty requires the proper development and function of the hypothalamic-pituitary-gonadal axis orchestrated by gonadotropin-releasing hormone (GnRH). GnRH neuron cells originate in the olfactory placode region, and migrate with olfactory axons to the hypothalamus during development(1-2). Pulsatile GnRH triggers the synthesis and release of pituitary gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The gonadotropins then stimulate gonadal steroidogenesis and gametogenesis in both sexes.

Idiopathic hypogonadotropic hypogonadism (IHH) constitutes one of the most common inherited forms of congenital hypogonadism(3). These patients present with absent puberty due to impaired GnRH secretion and/or action, low serum sex steroids and gonadotropins(4-6). Kallmann syndrome (KS)—IHH and anosmia—results from impaired GnRH neuron migration and olfactory bulb dysgenesis. Associated anomalies include synkinesia, ataxia, visual abnormalities, hearing loss, dental agenesis, craniofacial defects, and renal agenesis(7). Inheritance may be autosomal dominant, autosomal recessive, and X-linked recessive, or it may be sporadic. Digenic disease has also been reported(8-10) and IHH/KS may be reversible in some cases(11).

At least one mutation has been reported in *KAL1*, *NR0B1*, *GNRHR*, *LEP*, *LEPR*, *PCSK1*, *FGFR1*, *FGF8*, *KISS1R*, *PROK2*, *PROKR2*, *CHD7*, *TAC3*, *TACR3*, and *GNRH1* genes(7,12). Among these, *FGFR1* (10%)(13), *GNRHR* (3%)(3,14-15), and *CHD7* (6%)(16)

are more frequently observed in normosmic IHH (nIHH); while *KAL1* (5%)(7,17-19), *FGFR1* (10%)(13,20), *PROKR2* (5%)(8,21), and *CHD7* (6%)(16) are more common in KS.

Recently, nasal embryonic LHRH-factor (NELF) was found to be differentially expressed in migratory GnRH neurons(22-23). Only two *NELF* mutations have been reported in IHH/KS. One was identified in nIHH, but neither *in vitro* analysis nor mutation screening of additional IHH/KS genes was performed(24). The other *NELF* mutation was identified in KS in a digenic pattern with *FGFR1*(9). No heterozygous intragenic *NELF* deletions have been described(25), and no *NELF* mutations supported by functional analysis, have been reported in monogenic IHH/KS. The purpose of this study was to determine whether IHH/KS is caused by *NELF* mutations solely or in combination with mutations in other known genes.

Materials & Methods

Patients

IHH was diagnosed in males ≥18 years with absent puberty, testosterone levels <100ng/dL (normal 300-1100) and low or normal serum gonadotropins. In females, IHH was defined as primary amenorrhea at ≥17 years with low estradiol (<30pg/mL) and low/normal gonadotropins(4-5). All patients had normal thyroid-stimulating hormone, thyroxin, cortisol and prolactin. No pituitary tumor was present by radiographic imaging. Complete IHH/KS was defined as the absence of puberty without thelarche (Tanner 1) in females and testes ≤3mL in males. Incomplete IHH/KS was defined as partial breast development in females and testes ≥4mL in males(4). Anosmia was defined using the University of Pennsylvania Smell Test, when available, or by history. Lymphoblastoid cell lines were generated as a source for DNA, RNA, and/or protein(4). All patients signed an informed consent approved by the Human Assurance Committee of the Medical College of Georgia.

Genomic Analysis

Genomic DNA from 168 unrelated IHH/KS patients was amplified by nested-PCR using *NELF* primers flanking the 15 exons/splice junctions (Table S1). DNA sequencing was performed as described previously(14). Putative mutations were confirmed and sequenced in ≥180 healthy controls. Four *NELF* nucleotide variants identified in three IHH/KS patients were screened in 372 ethnically-matched controls. In the patient with bi-allelic *NELF* mutations, PCR products were cloned using the TA-Cloning Kit (Invitrogen, Carlsbad, California) to determine if they were on the same allele(16). To exclude digenic disease in patients with *NELF* mutations, the protein-coding regions and splice junctions were sequenced for other known IHH/KS genes—*CHD7, FGF8, FGFR1, PROK2, PROKR2, TAC3, TACR3, KAL1, GNRHR, GNRH1*, and *KISS1R* (primers are available upon request).

Splice Mutant Detection

Intronic mutations were analyzed using ESE Finder Version 3(http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi) to determine any effect upon predicted SR protein binding sites involved in splicing(26). Sequence was also analyzed at the Berkley Drosophila Genome Project site (http://www.fruitfly.org/seq_tools/splice) to predict possible effects on 5' and 3' splicing consensus sequences. RT-PCR was used to determine if the mutations affected splicing. Total RNA was extracted from lymphoblastoid cells using TriReagent (Molecular Research Center; Cincinnati, Ohio) and subjected to SuperScript™III One-Step RT-PCR with Platinum®*Taq* (Invitrogen, Carlsbad, California) using gene-specific primers with appropriate negative and positive controls. PCR products were electrophoresed, purified, cloned using the TA Cloning Kit, and sequenced.

Quantitative real-time RT-PCR

Lymphoblast RNA was used for RT-qPCR with Superscript II Platinum SYBR Green One-Step qRT-PCR kit(27). A standard curve was constructed with known amounts of human *NELF* cDNA (primers 5'AACGGCGCAGATCACCTG3' and 5'TGCGGGCTTCCTAATGCT3') and for *PPIA*, encoding cycolphilin-A (primers 5'CCTAAAGCATACGGGTCCTG3'and 5'GCCATCCAACCACTCAGTCT3'). *NELF* RT-qPCR was performed in triplicate, normalized to *PPIA*, and analyzed using the C_T method with a mixed model ANOVA.

Protein Expression

Western blots were performed on lymphoblast protein using standard methods(28) with our polyclonal NELF antibody(27) and a β-actin antibody to control for loading differences. NELF/β-actin band intensity was quantitated by densitometry(27).

Results

Three of 168 (1.8%) IHH/KS patients demonstrated *NELF* mutations-two males with KS and one male with nIHH. These included: one heterozygous missense mutation (c.757G>A; p.Ala253Thr), one heterozygous splice mutation (c.1160-13C>T), and compound heterozygous intronic mutations (c.629-21C>G; c.629-23G>C), which were not present in the NCBI SNP database or 372 controls (Table 1; Figure 1A). Additionally, 11 novel polymorphisms were identified (Table S2).

The functional significance of four specific nucleotide changes was investigated for *in vitro* evidence of impaired function. The first patient, nIHH male C54, had compound heterozygous *NELF* mutations (c.629-21C>G/c.629-23G>C) as determined by sequencing cloned PCR-products (Table 1; Figure 1A). Analysis of lymphoblast NELF protein consistently demonstrated a ~50% reduction compared to control (Figure 2). ESE-Finder analysis predicts that c.629-21C>G will create new SF2/ASF and SC35 binding sites for SR proteins, while the c.629-23G>C will create a new SF2/ASF binding site. However, no reduced mRNA expression or altered splicing was observed in lymphoblasts (not shown). This patient had no other associated anomalies or affected family members. Importantly, he had no coexistent mutations in *CHD7*, *FGF8*, *FGFR1*, *PROK2*, *PROKR2*, *TAC3*, *TACR3*, *KAL1*, *GNRHR*, *GNRH1*, or *KISS1R*.

The second patient (KS male C7) had a heterozygous c.757G>A (p.Ala253Thr) mutation (Figure 1; Table 1) affecting a completely conserved Ala253 residue (Figures S1-4). Using multiple sequence alignment (ESPRESSO)(29), a protein model for the N-terminus was constructed(30). Both SSPIDER(31) and INTERPROSURF analysis (Figure S4) suggest functional importance for Ala253; and SIFT(32) predicts a deleterious effect for p.Ala253Thr. Although p.Ala253Thr did not alter splicing or quantitative mRNA expression (not shown), lymphoblast protein expression was consistently reduced by 50% *in vitro*.

This p.Ala253Thr mutation was identified in a male with sporadic KS, unilateral renal agenesis, and partial pubertal development. He also had a *KAL1* deletion (c. 488_490delGTT;p.Cys163del) (Table 1; Figure 1B) we characterized previously(17). This in-frame deletion removes a fully conserved cysteine residue in the anosmin-1 protein encoded by *KAL1* (Figure S1C,D). The KS proband with *NELF/KAL1* mutations had no mutations in *CHD7*, *FGF8*, *FGFR1*, *PROK2*, *PROKR2*, *TAC3*, *TACR3*, *GNRHR*, *GNRH1*, or *KISS1R*.

The third *NELF* mutation analyzed *in vitro* was a heterozygous c.1160-13C>T in intron 10 identified in male KS patient C68 (Figure 1A; Table 1). He had completely absent puberty,

bilateral cryptorchidism, no other anomalies, and no other affected family members. This mutation was predicted to alter the location of an SF2/ASF binding site and result in the loss of an SRp40 binding site, both of which could disrupt normal splicing(26). The weak splice acceptor site score of 0.52 predicts that this 3' splice site may be susceptible to alternative splicing events; in fact, exon 10 skipping was demonstrated by RT-PCR and subcloning (Figure 3). The mutation introduces two aberrant out-of-frame AA, causing a frameshift and a premature termination codon at residue 378 located 9bp downstream of the junction between exons 9 and 11. The resulting NELF protein is predicted to be truncated >28% of the C-terminus. The altered transcript was seen in 4/30 clones from patient C68, but not in 100 clones obtained by RT-PCR from three unrelated controls. The intronic mutation did not quantitatively affect mRNA or protein expression (not shown). However, this genomic c. 1160-13C>T variant was not seen in the NCBI SNP database or in 372 ethnically-matched controls, arguing against a polymorphism.

This patient also was found to have a novel, heterozygous *TACR3* nonsense mutation p.Trp275X not seen in 180 controls (Figure 1C; Table 1). Trp275 lies within a cytoplasmic domain between the 5^{th}-6^{th} transmembrane domains of this G-protein coupled receptor(33), thereby predicting the loss of 191AA from codons 275-465 and truncating ~40% of the C-terminus (Figure 1C). He had no mutations in *CHD7, FGF8, FGFR1, PROK2, PROKR2, TAC3, KAL1, GNRHR, GNRH1,* or *KISS1R*. Unfortunately, in all three probands with *NELF* mutations, no other family members were available for *de novo* or segregation analysis.

Discussion

Our findings indicate that *NELF* is likely to be causative in IHH/KS. Previously, Miura *et al*(24) demonstrated a heterozygous c.1438A>G (p.Thr480Ala) *NELF* variant in 1/65 IHH patients based upon sequence AY_255128 (now revised to c.1432A>G ;p.Thr478Ala from NP_056352). Since Thr478 was only partially conserved (Figure S1B) and no *in vitro* analysis was performed, its functional significance is unknown(24). No other IHH/KS genes were studied, so digenic disease cannot be excluded.

One heterozygous *NELF* splice mutation (c.1159-14_22del) has been described(9). However, the only KS individual within the family also had a heterozygous *FGFR1* mutation (p.Leu342Ser), suggesting digenic disease(9). This *NELF* deletion was associated with exon 10 skipping, but was not sufficient to cause KS alone(9). Therefore, no human *NELF* mutations, supported *in vitro*, and without mutations in a second gene, have been reported to cause IHH/KS.

In the present study, 3/168 (1.8%) of IHH/KS patients had *NELF* mutations demonstrating impaired function *in vitro*, which is similar to *GNRHR*(14) and greater than *KISS1R* mutations(34) in nIHH patients. To exclude digenic disease, sequencing of 11 additional genes (*CHD7, FGF8, FGFR1, PROK2, PROKR2, TAC3, TACR3, KAL1, GNRHR, GNRH1*, and *KISS1R*) was performed.

Compound heterozygous *NELF* mutations in a nIHH male quantitatively reduced protein expression ~50%, but unexpectedly did not reduce mRNA expression. mRNA/protein discordance can occur by "leaky" expression of splice variants(35), altered expression of proteins with multiple isoforms(36), altered protein translation rate(37), posttranslational modifications(38), and has been seen in microarray/proteomics comparisons(39-40). The exact mechanism by which our mRNA/protein discordance occurs is unknown. Since these intronic sequence variants introduce two additional SR protein binding sites important in constitutive splicing, their perturbation could affect alternative splicing in a tissue and developmentally-specific fashion(26). Alternatively, human NELF and its rat

ortholog(24,27,41) have multiple isoforms, which could contribute. We hypothesize that biallelic *NELF* mutations are necessary for the phenotypic manifestation of IHH. Our nIHH male had no additional mutation in 11 known IHH/KS genes studied. We believe that these are the first reported biallelic *NELF* mutations.

Two additional heterozygous *NELF* mutations occurred in unrelated KS patients in a digenic pattern. In a KS male, heterozygous *NELF*/hemizygous *KAL1* mutations were identified that were not observed in 372 ethnically-matched controls. The p.Ala253Thr *NELF* missense mutation resulted in a drastic AA substitution from a hydrophobic to hydrophilic residue. Multiple *in silico* methods also suggest that his mutation is likely to be deleterious. These include complete conservation of Ala253 (Figures S1-S4), SIFT analysis, and protein modeling. Furthermore, protein expression was markedly reduced *in vitro*, providing additional support for a deleterious effect. This patient with the p.Ala253Thr *NELF* missense mutation also had a hemizygous *KAL1* deletion of the completely conserved Cys163 within the whey-acidic-protein (WAP) domain that forms a disulphide bridge with Cys134 of anosmin-1 (Figure S1C,D)(42). Unilateral renal agenesis in this patient is likely related to this deleterious *KAL1* mutation(43).

The third KS male was heterozygous for both *NELF* and *TACR3* nonsense mutations. The *NELF* mutation disrupted splicing, deleting exon 10, and is predicted to cause protein truncation. Although observed in a subset of clones, this splice mutant was not seen in 100 control clones; and the mutation was not observed in 372 ethnically-matched controls. The low frequency of this altered transcript suggests potential mRNA instability, but we cannot exclude the possibility that this splice variant could be a rare sequence variant.

The combination of heterozygous *NELF/TACR3* mutations provides intriguing observations into KS patients with digenic disease(33). To date, homozygous *TACR3* mutations have only been identified in nIHH. p.Trp275X is not only the first nonsense *TACR3* mutation reported, it is also the first heterozygous *TACR3* mutation identified in KS in a digenic pattern with *NELF*. p.Trp275X is predicted to abolish the 6^{th}-7^{th} transmembrane domains with truncation the C-terminus of the neurokinin B receptor-3 encoded by *TACR3* (Figure 1C)(33). Disruption of G-protein transmembrane domains commonly impairs signal transduction in nIHH/KS genes *GNRHR*(3), *KISS1R*(34), *TACR3*(33), and *PROKR2*(44).

Perhaps the combination of a heterozygous *TACR3* nonsense mutation with a heterozygous *NELF* truncation mutation could cause a more severe phenotype, like our KS patient with completely absent puberty and bilateral cryptorchidism (Table 1). It is interesting that our *NELF* splice mutant and the one reported previously(9) resulted in exon 10 skipping, but neither alone was sufficient to cause KS—it required a mutation in a second gene.

Two unrelated KS patients in this study demonstrated previously unreported patterns of *NELF/KAL1* and *NELF/TACR3* mutations. We hypothesize that mutations in two loci exacerbate phenotype expressivity by synergistic heterozygosity. Synergistic heterozygosity refers to phenotypic expression caused by several partial protein defects in ≥1 pathway(45) —quite conceivable for known IHH/KS genes *KAL1* and *TACR3*. Synergistic heterozygosity has been described in metabolic disorders(45-46) and primary pulmonary hypertension(47). In addition to KS(8-10), mutations in more than one gene have been reported in other diseases such as retinitis pigmentosa, epidermolysis bullosa, and deafness(48-50).

Overall, NELF lacks homology to any known protein sequence except for AA209-528 used for protein modeling; and has no N-terminal signal peptide(24). We have demonstrated intranuclear expression in immortalized GnRH neurons and identified a functional nuclear localization signal and putative zinc fingers, suggesting that it could be a transcription

factor(27). It is tempting to speculate that NELF might bind the *KAL1* and/or *TACR3* promoters, although this has yet to be determined experimentally. However, NELF is involved in GnRH neuron migration in mouse(22,27), zebrafish(51), and human as determined in this study by the demonstration of mutations in KS.

Human *NELF* has been localized to chromosome 9q34.3 where a deletion syndrome has been described in two males with abnormal genitalia and an anosmic female(52). The deletion encompasses *NELF*, thereby implicating *NELF* in the partial KS phenotypes of these individuals. Based upon our findings and the *NELF* deletion within 9q34.3, we propose that *NELF* loss-of-function might be a potential mechanism for IHH/KS.

In summary, three novel human *NELF* mutations were identified in IHH/KS for a prevalence of about 2%. We describe the first compound heterozygous *NELF* mutation reported in a nIHH male without mutations in 11 known IHH/KS genes. In addition, a previously unreported association of *NELF/KAL1* and *NELF/TACR3* was identified in KS. Our findings suggest that mutation of one *NELF* allele may not be sufficient to result in disease unless there is an additional mutant *NELF* allele or coexistent mutation in another gene. These findings, along with several previous reports of *NELF/FGFR*(9) and *PROKR2/KAL*(8,10), highlight the importance of biallelic mutations in IHH/KS and warrant future consideration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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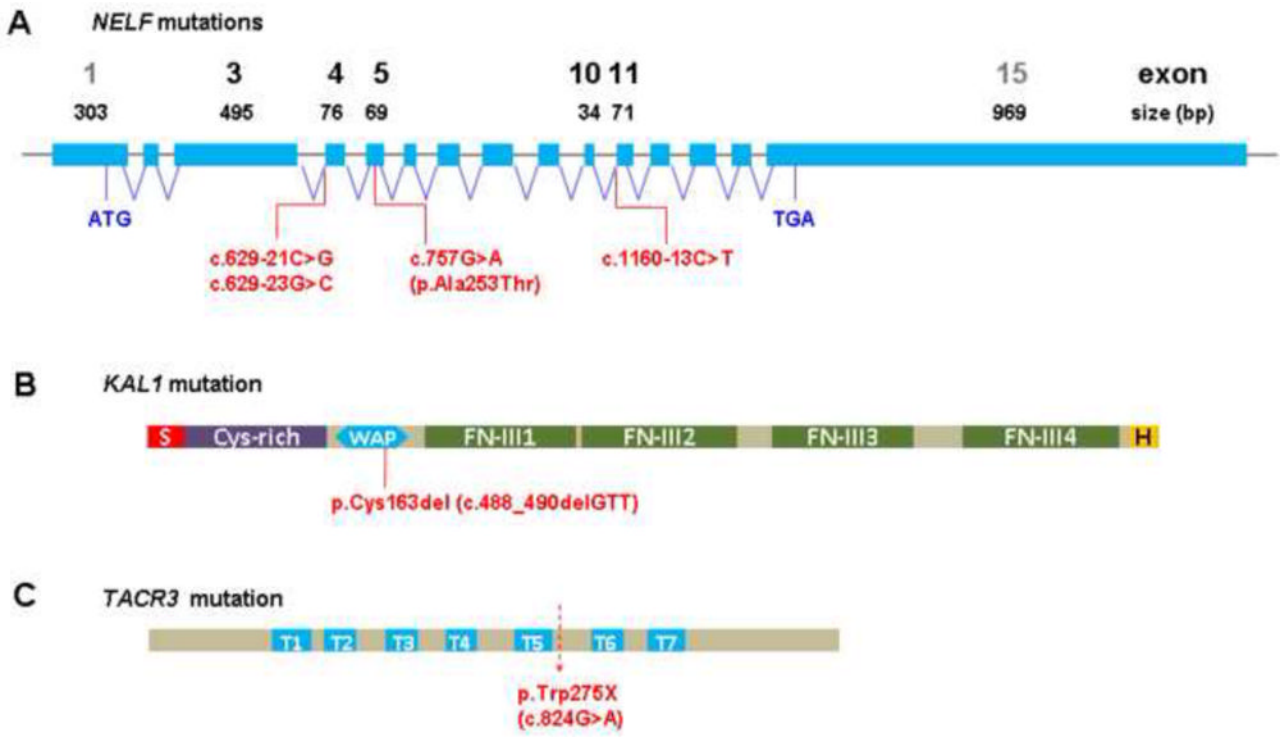
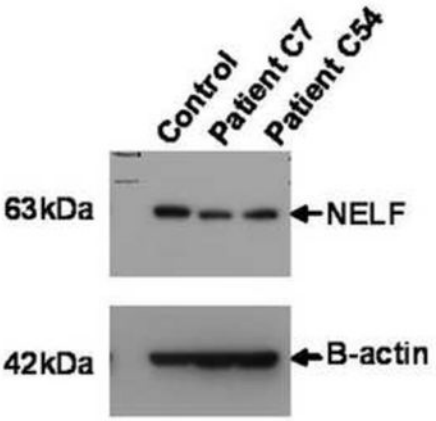


Figure 1. Location and conservation of *NELF*, *KAL1*, and *TACR3* mutations

(A) Exon and intron structure of the 58 kb *NELF* gene (NM_015537) with locations of human missense and intronic mutations identified in sporadic IHH and KS patients. Important exons shown as blue rectangles to scale are numbered along with their size in basepairs (bp). The locations of start and stop codons are to scale, but the sizes of introns are not to scale.

(B,C) Graphical views of *KAL1*-encoded anosmin-1 (NP_000207) and TACR3 (NP_001050) proteins with known domains. Shown is the single AA cysteine in frame *KAL1* deletion in the whey-acidic-protein domain (WAP) in B—although the annotation (c. 487_489delTGT) was incorrect in our previous report (17). Also shown is the *TACR3* truncation mutation p.Trp275X within a cytoplasmic domain between the 5th and 6th transmembrane domains in C). The relative size between anosmin-1 and TACR3 proteins and the size and locations of depicted domains are to scale. Abbreviations include S=signal peptide; Cys-rich (cysteine-rich), FN-III=fibronectin III; H=histidine; T1-T7=transmembrane 1-7.



Antibody	Control	Pt. C7	Pt. C54
NELF	2075	1396	1435
B-actin	4152	5018	5083
NELF/actin	0.4998	0.2782	0.2823
Normalized to Control	1.000	0.5566	0.5648

Figure 2. Functional protein analysis of human *NELF* mutations

Western blot analysis is shown using the anti-NELF antibody (27), which recognizes the ~63 kDa NELF protein in lymphoblastoid cell lines extracts. β-actin was used as internal loading control (~42kDa). Lane 1 represents a normal male control, whereas lanes 2-3 represent individuals with *NELF* mutations. Additional controls and other mutations did not alter protein expression, and are not shown. The westerns were performed three times, and the results were consistently the same. Protein expression for patient C68 was not altered (not shown).

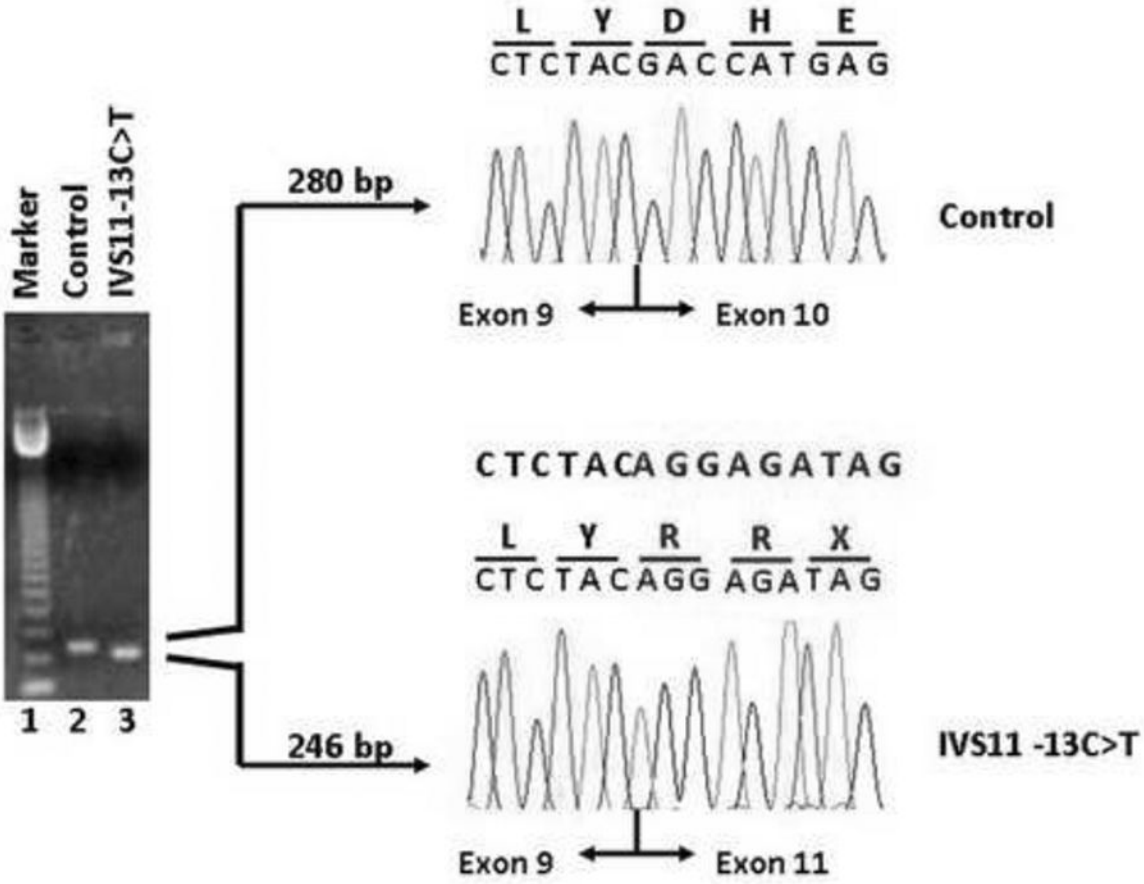


Figure 3. *NELF* Splice Mutant

Subcloning and sequencing analysis of RT-PCR products of *NELF* exons 8-12 from patient and control lymphoblastoid RNA confirms that the human *NELF* c.1160-13C>T mutation causes aberrant exon 10 skipping. Splicing patterns are compared between a normal control and the mutant by cloned cDNA sequencing (the WT or mutant band is a cloned fragment). An expected *NELF* product of 280 bp including exons 9-10 is observed in the control (lane 2) whereas an abnormal product of 246 bp skipping exon 10 (34 bp) is observed in a KS patient with c.1160-13C>T (lane 3). Exon 11 nucleotides and out-of-frame novel AA sequence are depicted in blue. A 123 bp DNA marker is shown in the first lane.

Table 1

***NELF* (NM_015537) mutations in sporadic patients with nHH/KS**

In patient C54, compound heterozygous mutations were present. In the two other patients with digenic disease, *NELF* mutations were heterozygous. The *KAL1* mutation was hemizygous and the *TACR3* mutation was heterozygous. Complete HH refers to the complete absence of sexual development, while incomplete HH indicates partial pubertal development. Testes size is given (normal is 15-25mL).

Patient	Gender and phenotype	Geographic origin	Exon/intron	Nucleotide change	AA Change	Mutation	Confirmatory method	Mutations in a second gene
C54	Male / nHH Incomplete HH Normosmia Testes: 12,15mL LH=3.2 mIU/mL FSH=1.2 mIU/mL	USA	Intron 2	c. 629-21C>G & c. 629-23G>C compound heterozygous	NA	Intronic	0/372 controls; decreased protein by western blot	None
C7	Male / KS Incomplete HH Anosmia Testes: 4, Cryptorchidism LH<1.5 mIU/mL FSH<3.6 mIU/mL Unilateral renal agenesis	USA	Exon 5	c.757G>A	p.Ala253Thr	Missense	0/372 controls; SIFT; protein modeling; decreased protein by western blot; impaired nuclear localization	*KAL1* del c.488_490delGTT (p.Cys163del)
C68	Male / KS Complete HH Anosmia Bilateral cryptorchidism LH=0.5 mIU/mL FSH=0.6 mIU/mL	USA	Intron 10	c. 1160-13C>T	NA	Intronic	0/372 controls; skips exon 10 in RT-PCR;	*TACR3* c.824G>A (p.Trp275X)

SIFT = sorting intolerant from tolerant; NA = not applicable.