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Research Article

Endocrinology

Idiopathic hypogonadotropic hypogonadism (IHH) due to defects of gonadotropin-releasing hormone (GnRH) secretion and/or action is a developmental disorder of sexual maturation. To date, several single-gene defects have been implicated in the pathogenesis of IHH. However, significant inter- and intrafamilial variability and apparent incomplete penetrance in familial cases of IHH are difficult to reconcile with the model of a single-gene defect. We therefore hypothesized that mutations at different IHH loci interact in some families to modify their phenotypes. To address this issue, we studied 2 families, one with Kallmann syndrome (IHH and anosmia) and another with normosmic IHH, in which a single-gene defect had been identified: a heterozygous *FGF receptor 1 (FGFR1)* mutation in pedigree 1 and a compound heterozygous *gonadotropin-releasing hormone receptor (GNRHR)* mutation in pedigree 2, both of which varied markedly in expressivity within and across families. Further candidate gene screening revealed a second heterozygous deletion in the *nasal embryonic LHRH factor (NELF)* gene in pedigree 1 and an additional heterozygous *FGFR1* mutation in pedigree 2 that accounted for the considerable phenotypic variability. Therefore, 2 different gene defects can synergize to produce a more severe phenotype in IHH families than either alone. This genetic model could account for some phenotypic heterogeneity seen in GnRH deficiency.

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# Digenic mutations account for variable phenotypes in idiopathic hypogonadotropic hypogonadism

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**Idiopathic hypogonadotropic hypogonadism (IHH) due to defects of gonadotropin-releasing hormone (GnRH) secretion and/or action is a developmental disorder of sexual maturation. To date, several single-gene defects have been implicated in the pathogenesis of IHH. However, significant inter- and intrafamilial variability and apparent incomplete penetrance in familial cases of IHH are difficult to reconcile with the model of a single-gene defect. We therefore hypothesized that mutations at different IHH loci interact in some families to modify their phenotypes. To address this issue, we studied 2 families, one with Kallmann syndrome (IHH and anosmia) and another with normosmic IHH, in which a single-gene defect had been identified: a heterozygous *FGF receptor 1 (FGFR1)* mutation in pedigree 1 and a compound heterozygous *gonadotropin-releasing hormone receptor (GNRHR)* mutation in pedigree 2, both of which varied markedly in expressivity within and across families. Further candidate gene screening revealed a second heterozygous deletion in the *nasal embryonic LHRH factor (NELF)* gene in pedigree 1 and an additional heterozygous *FGFR1* mutation in pedigree 2 that accounted for the considerable phenotypic variability. Therefore, 2 different gene defects can synergize to produce a more severe phenotype in IHH families than either alone. This genetic model could account for some phenotypic heterogeneity seen in GnRH deficiency.**

## Introduction

Genetic analyses of idiopathic hypogonadotropic hypogonadism (IHH), an important human disease model with implications for the discovery of genes responsible for human puberty, have provided considerable insight into the genes that control sexual maturation. IHH is a clinically and genetically heterogeneous disorder resulting in gonadotropin-releasing hormone (GnRH) deficiency that can be inherited as an X-linked, autosomal recessive, or autosomal dominant trait. IHH has been considered to be a monogenic disorder with several loci identified to date: *Kallmann syndrome 1 sequence (KAL1)* (1–3), *FGF receptor 1 (FGFR1)* (4), *prokineticin 2 (PROK2)*, and *prokineticin receptor 2 (PROKR2)* (5) underlie cases of Kallmann syndrome (KS), while *gonadotropin-releasing hormone receptor (GNRHR)* (6), *FGFR1* (7), and *G protein-coupled receptor 54 (GPR54)* (8, 9) underlie normosmic IHH (nIHH). Additionally, *nasal embryonic LHRH factor (NELF)* has been implicated in the pathogenesis of KS (10). Despite these advances, conundrums remain in understanding the genetic basis of IHH. For example, there is a puzzling clinical heterogeneity of the reproductive and

nonreproductive phenotypes both within and across IHH families carrying identical single gene mutations (4, 6, 11–14). Therefore, a given genotype at a single locus cannot reliably predict the phenotypic manifestations in any given member of affected families. Additionally, some mutations in genes accounting for IHH, especially *FGFR1*, apparently demonstrate incomplete penetrance (4). Finally, defects in the identified genetic loci account for only a small percentage (<30%) of cases. Thus, it is likely that other major IHH loci remain to be discovered and/or that the remaining (>70%) cases are caused by the interplay of several contributing genes.

We hypothesized that IHH often involves defects in more than 1 gene. Herein we report evidence of IHH caused by the interaction of 2-gene defects (*FGFR1* and *NELF* in pedigree 1 and *GNRHR* and *FGFR1* in pedigree 2). In addition, *in vitro* biochemical characterization of *FGFR1* and *NELF* mutants is provided.

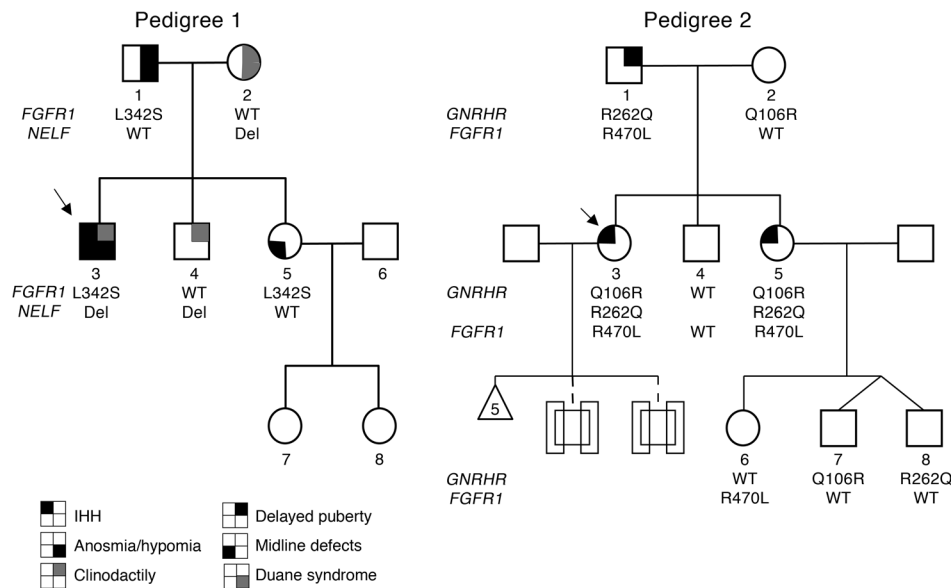
## Results

**Pedigree 1.** Pedigree 1 has several affected members (Figure 1). The proband (no. 1-03) was referred to an endocrinologist at age 21 for failure to undergo puberty. His presentation was consistent with a severe KS phenotype. He was unvirilized, had eunuchoidal proportions (height, 176 cm; span, 185 cm), bilateral gynecomastia, microphallus, prepubertal testes (2 ml; normal, >12 ml), a repaired cleft lip/palate, and clinodactyly. His serum gonadotropin levels were undetectable, testosterone (T) was 1 nmol/L, and inhibin B 74 pg/ml; otherwise, he had normal pituitary function and brain imaging.

**Nonstandard abbreviations used:** D3, immunoglobulin-like domain 3; *FGFR1*, *FGF receptor 1*; GnRH, gonadotropin-releasing hormone; *GNRHR*, gonadotropin-releasing hormone receptor; IHH, idiopathic hypogonadotropic hypogonadism; *KAL1*, Kallmann syndrome 1 sequence; KS, Kallmann syndrome; *NELF*, nasal embryonic LHRH factor; nIHH, normosmic IHH; SPR, surface plasmon resonance; T, testosterone.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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**Figure 1** Identification of *FGFR1* (p.L342S) and *NELF* (8-bp intronic deletion) mutations in pedigree 1; identification of *GNRHR* [p.Q106R] and [p.R262Q] and *FGFR1* (p.R470L) mutations in pedigree 2. Only subjects harboring 2 gene defects have IHH. Proband is identified by arrows; circles denote females, squares denote males. Del, *NELF* intronic deletion.

Formal testing revealed hyposmia (score of 29/40, below fifth percentile for his age) (15). Two subsequent years of gonadotropin therapy induced full virilization and sperm production. His father (no. 1-01) had a history of delayed puberty (growth spurt and full virilization after age 17) and congenital anosmia (score of 13/40), and his adult serum T was 18.1 nmol/l. The proband's mother (no. 1-02) had clinodactyly and Duane ocular retraction syndrome and was menopausal. The proband's sister (no. 1-05) exhibited midline defects, including a bifid nose and high arched palate; the brother (no. 1-04) exhibited clinodactyly only. The mother and both siblings had normal puberty and a normal sense of smell as determined by formal testing.

**Mutational analysis of the *FGFR1* gene.** The proband (no. 1-03) carries a unique heterozygous mutation (c.1025 T→C) in exon 7 predicted to substitute a leucine for serine at position 342 (p.L342S) in the immunoglobulin-like domain 3 (D3) of *FGFR1* (Figure 1, Figure 2, A and B, and Figure 3G). This change was also found in the affected father (no. 1-01) and the affected sister (no. 1-05) (Figure 1) but not in 200 white controls.

**Structural and biochemical analysis of the L342S mutation implicate a loss of *FGF8b* signaling through *FGFR1c* in the pathology of *KS/IHH*.** The isoforms *FGFR1b* and *FGFR1c* are generated by alternative splicing of exons 8A and 8B, respectively (16). To date, *FGFR1* mutations causing IHH have only been identified in exon 8B (17), implicating *FGFR1c* in the pathogenesis of IHH. Because L342 is highly conserved among the "c" splice isoforms of *FGFR1*–3 across species (Figure 2B), we used the *FGFR2c*-*FGF8b* crystallography model (18) to study L342S. The corresponding amino acid in *FGFR2c*, L343, is a key constituent in the hydrophobic

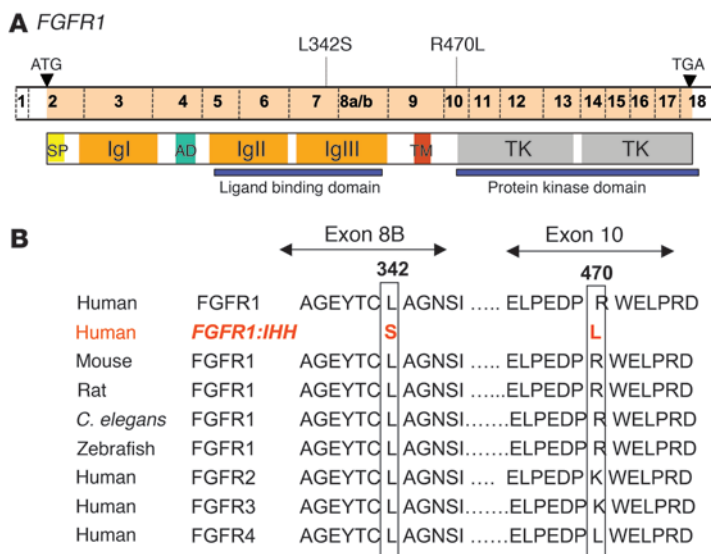
groove of D3 and is extensively engaged by *FGF8b* (18) (Figure 3G). This leucine accounts for the unique binding specificity of *FGF8b* for the "c" isoforms of *FGFR1*–3. Consistent with these structural data, surface plasmon resonance (SPR) analysis revealed a dramatic loss (20-fold) in the affinity of the L342S mutant for *FGF8b* (Figure 3, C and F), with only a small decrease (2-fold) in affinity for *FGF1* (Figure 3, A and D) and (3-fold) *FGF2* (Figure 3, B and E).

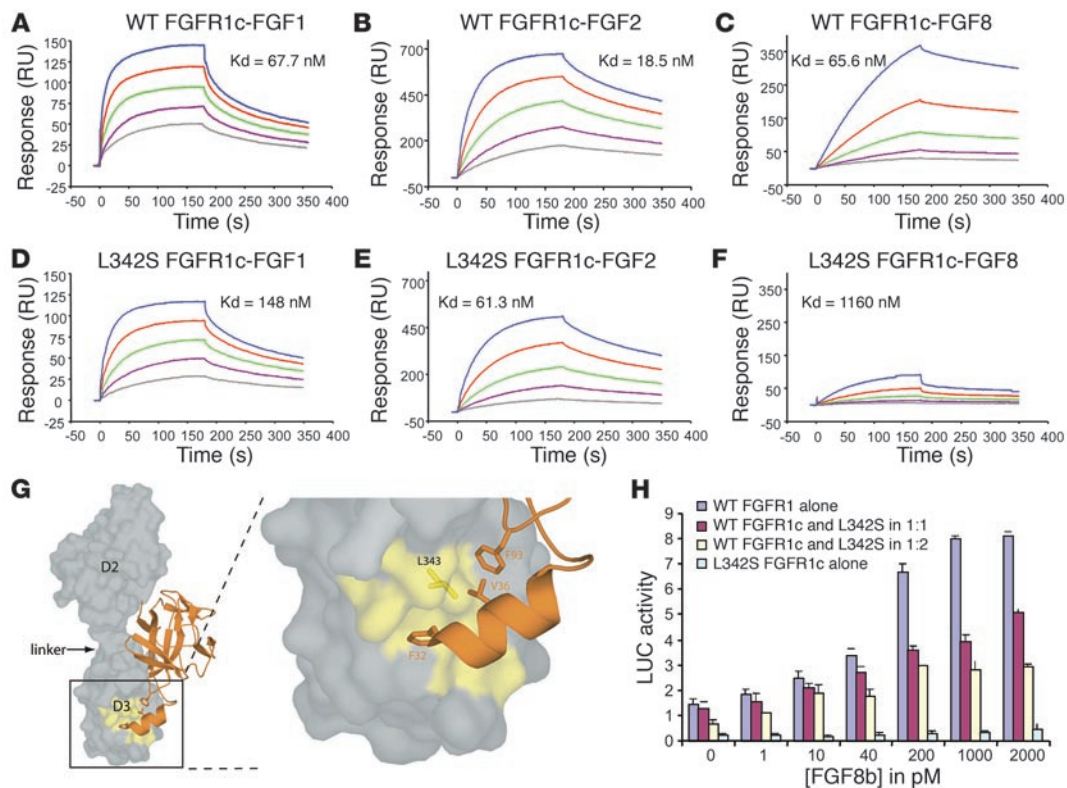
**L342S *FGFR1c* missense mutation is a loss-of-function mutation.** L6 myoblasts transiently expressing WT *FGFR1c* were treated with *FGF8b*, which induced a 6-fold increase in LUC reporter gene expression (Figure 3H). In agreement with SPR results, the L342S *FGFR1c* was silent when expressed alone. As the *KS* subject harbored a heterozygous L342S mutation, we further coexpressed the WT *FGFR1* and L342S in 1:1 and 1:2 ratios. The results are compatible with the hypothesis that this mutant acts as a dominant-negative mutation.

The variable degree of sexual maturation among family members carrying the same *FGFR1* mutation led to further candidate

**Figure 2**

Schematic showing location of the 2 *FGFR1* mutations and conservation of L342 and R470 residues across species and *FGFRs*. (A) The *FGFR1* gene is located on chromosome 8p. *FGFR1* contains 18 exons with intervening introns not drawn due to scale. SP, signal peptide; TM, transmembrane domain; TK, tyrosine kinase domain. (B) Comparison of L342 and R470 across species and within the *FGFR* family.



**Figure 3**

The L342S mutation dramatically reduces affinity of FGFR1c for FGF8b, implicating decreased FGF8b/FGFR1c signaling in the etiology of KS/IHH. (A–F) The L342S mutation reduces the affinity of FGFR1c for FGF8b. Varying concentrations of WT (A–C) or L342S FGFR1c mutant (D–F) were injected over a CM5 chip onto which FGF1 (A and D), FGF2 (B and E), and FGF8b (C and F) were immobilized. Analyte concentrations are indicated as follows: 31.25 nM in gray, 62.5 nM in violet, 125 nM in green, 250 nM in red, 500 nM in blue. (G) The location of L343 in FGFR2c, the residue corresponding to L342 in FGFR1c, is mapped onto the FGF8b-FGFR2c structure (18). The L342S mutation should weaken key hydrophobic contacts between F32, V36, and F93 of FGF8b and D3 of FGFR. Gray: molecular surface of FGFR; orange: FGF8b. The side chains of selected residues are shown. The molecular surface of the hydrophobic groove of FGFR D3 (yellow) is rendered transparent so that the side chain of L343 (the residue corresponding to L342 of FGFR1c) is visible. (H) L342S FGFR1 heterozygous mutation is a loss-of-function mutation. WT and L342S FGFR1c were transiently transfected into L6 myoblasts with an FGFR1-responsive osteocalcin promoter luciferase construct. FGF8b treatment of WT FGFR1c induced a 6-fold increase in LUC reporter gene expression, while the L342S FGFR1c alone remained silent. The coexpression of the WT and L342S FGFR1c suggests that this mutation acts as a dominant negative.

gene screening and the identification of an additional deletion in *NELF* in the severely affected proband.

**Mutational analysis of the *NELF* gene.** A heterozygous 8-bp deletion ending 14 bp before exon 10 (c.1159-14\_22del) was identified in the proband (no. 1-03), his mother (no. 1-02), and his brother (no. 1-04) (Figure 4A). This deletion was not observed in 384 white controls.

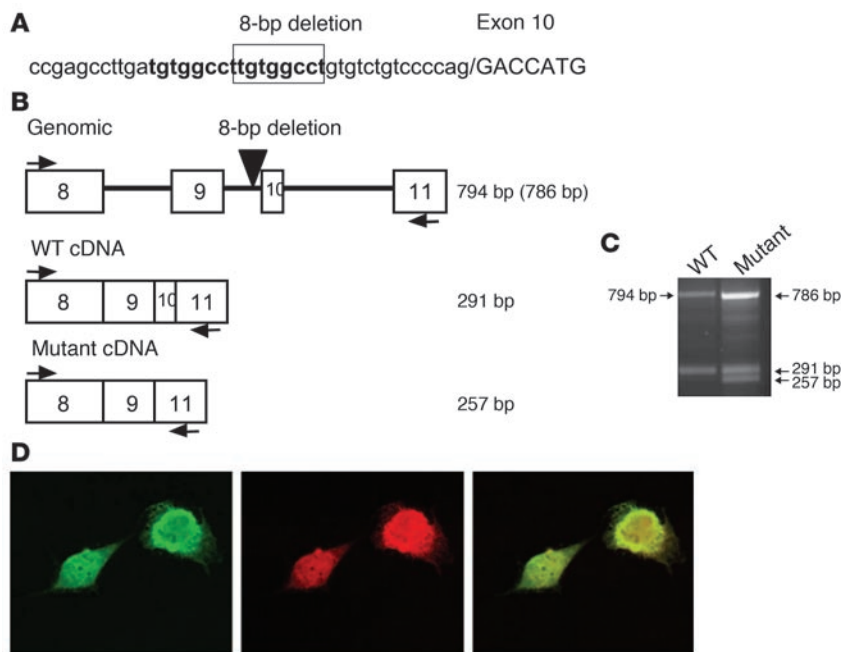
**RT-PCR.** The HEK-293 cells transfected with the WT plasmid expressed *NELF* exons 8–11 at the expected size (291 bp). However, cells transfected with the 8-bp intronic deletion plasmid expressed an additional 257-bp splice form of *NELF* mRNA, lacking exon 10 (Figure 4C). The missplicing of mRNA lacking exon 10 results in a premature stop codon that predicts a truncated *NELF* protein product (p.Y376X) rather than the full-length product, which comprises 528 residues.

**Immunohistology of FNC-C4 cells.** Colocalization of both *NELF* and GnRH1 in FNC-B4 cells was demonstrated by immunohistochemistry (Figure 4D) and RT-PCR (data not shown).

**Genotype-phenotype correlations.** Proband no. 1-03 exhibited a severe KS phenotype with absent puberty, microphallus, hyposmia, undetectable serum luteinizing hormone and follicle stimulating

hormone levels, hypogonadal T levels, and low inhibin B levels. He also had clinodactyly and cleft lip and palate. He harbored both a paternally derived heterozygous *FGFR1* mutation (c.1025 T→C, p.L342S), resulting in weaker binding to FGF8b, and a maternally derived heterozygous 8-bp intronic deletion of *NELF*, resulting in a splicing defect of exon 10 and premature stop codon (Figures 1–4). While a single gene defect was associated with an attenuated phenotype (i.e., delayed puberty and anosmia in the father carrying L342S only), 2 mutant genes/gene products (FGFR1 and *NELF*) synergized to produce a more severe KS phenotype.

**Pedigree 2.** All members of the second, previously reported (14) pedigree were normosmic by formal smell testing, including 2 sisters affected with nIHH (Figure 1). The proband (no. 2-03) presented at 17 with primary amenorrhea, no breast development, short fourth metacarpals, and osteoporosis. She had undetectable serum gonadotropin and estradiol (E2) levels but otherwise normal pituitary function and cranial imaging. As previously reported, pulsatile GnRH induced ovulation in the proband, but she had 5 consecutive miscarriages (14). Her sister also presented at 18 with primary amenorrhea, absent breast development, and scoliosis. She had 2 success-



**Figure 4**

A 8-bp intronic *NELF* deletion results in missplicing of exon 10. (A) Direct DNA sequencing of cloned PCR products of the proband's genomic DNA revealed a heterozygous 8-bp deletion in intron 9. This deletion is part of a direct 8-bp repeat (tgtggcct) and occurs 14 bp upstream of the exon 10 acceptor (5') splice site. The lower case part of the sequence indicates introns while the upper case indicates exons. (B) Predicted cDNAs in the exon 8–11 region of the *NELF* gene. The proband was found to have an 8-bp deletion in intron 9 (black triangle), 14 bp upstream of exon 10. (C) The result of RT-PCR using HEK-293 cell mRNA from cells transfected with WT *NELF* genomic construct containing exons 8–11 and the mutant *NELF* construct (8-bp deletion in intron 9). The HEK-293 cells transfected with the WT *NELF* construct show a normally spliced *NELF* exon 8–11 RT-PCR product, corresponding to the predicted size of 291 bp. The cells transfected with the mutant *NELF* construct, however, show an additional band of 257 bp corresponding to the expected size of a transcript lacking exon 10. The RNA from cells transfected with either WT or mutant genomic construct show an additional product of 794 bp (786 bp for the mutant construct), reflecting PCR amplification of the residual plasmid DNA. (D) Colocalization of *NELF* and *GnRH1* in the olfactory epithelial cell line FNC-B4 by immunohistochemistry. Original magnification,  $\times 150$ .

ful pregnancies on gonadotropin therapy. Her daughter (no. 2-06) underwent normal puberty and has scoliosis, and male twins (nos. 2-07 and 2-08) were born without cryptorchidism or microphallus and have yet to undergo puberty (10 years old). The proband's father has a history of delayed puberty, scoliosis, and bilateral hearing loss. The brother was born with a cardiac septal defect and 3 fused cervical bones and went through normal puberty. The mother is menopausal but had normal reproductive function.

***GNRHR* mutational analysis.** A compound heterozygous mutation in *GNRHR* [Q106R] and [R262Q] was identified in the proband (no. 2-03) and her sister (no. 2-05) (14). Both exhibited severe nIHH with absent puberty. Their father (no. 2-01) with a history of delayed puberty was heterozygous for R262Q, while the mother with no reproductive phenotype (no. 2-02) was heterozygous for Q106R. In addition, the brother (no. 2-04) and the niece (no. 2-06) were WT, while the twins (nos. 2-07 and 2-08) were heterozygous for Q106R and R262Q, respectively (Figure 1). In 1997, de Roux et al. described 2 siblings with partial nIHH as evidence by spontaneous testicular growth and active spermatogenesis in the male and

Tanner IV breast development in the female carrying the same compound heterozygous [Q106R] and [R262Q] mutation in the *GNRHR* gene. In vitro studies demonstrated that each mutant, [Q106R] and [R262Q], resulted in loss of function (6). The variable expressivity ranging from absent puberty to a partial puberty across families carrying the same compound heterozygous *GNRHR* mutation led to further candidate gene screening, which identified an additional *FGFR1* mutation in our pedigree.

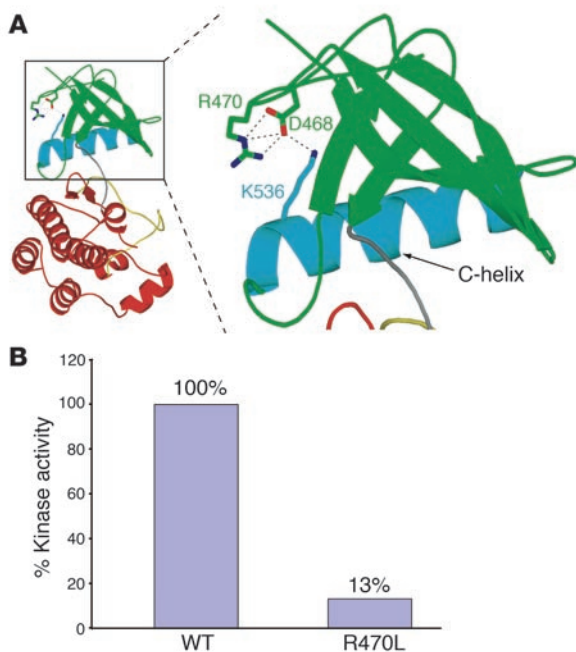
***FGFR1* mutational analysis.** We identified a heterozygous mutation in *FGFR1* (c.1409 G→T) in exon 10 in both nIHH sisters. This nucleotide change is predicted to substitute an arginine for leucine at position 470 (p.R470L) (Figure 2A). Screening of the entire pedigree revealed this change only in the father with delayed puberty (no. 2-01) and the unaffected niece (no. 2-06) (Figure 1). R470 is conserved across species (Figure 2B), and R470L was not detected in 200 white controls.

***The R470L mutation reduces the tyrosine kinase activity of FGFR1.*** In the *FGFR1* kinase structure, arginine 470 (R470) lies at the junction between the kinase domain and the juxtamembrane region (Figure 2A). The side chain of R470 makes 3 hydrogen bonds with D468; the latter engages in a hydrogen bond with K536 from the  $\alpha$ C helix (Figure 5A) (19). Crystal structures of unphosphorylated and phosphorylated kinase domains show distinct and reversible movements of the  $\alpha$ C helix during the kinase activation/inactivation cycle. R470 both facilitates the conformation of the juxtamembrane/kinase region and contributes to proper  $\alpha$ C positioning. Therefore, the R470L mutation should negatively impact the tyrosine kinase activity of *FGFR1*. Indeed, comparison of WT *FGFR1* and the R470L mutant revealed a marked decrease in the tyrosine kinase activity in the mutant, indicating a loss of function (Figure 5B).

***Genotype-phenotype correlations.*** The sisters severely affected with nIHH harbor both a compound heterozygous mutation in the *GNRHR* gene [Q106R] and [R262Q] and a heterozygous *FGFR1* mutation (R470L) — therefore a triallelic pattern of inheritance. The additional *FGFR1* mutation could explain the variable phenotypic expressivity seen between pedigrees harboring the same compound heterozygous *GNRHR* mutation (6). Interestingly, the father (no. 2-01), with a combination of 2 heterozygous mutant alleles (*GNRHR* and *FGFR1*), had delayed puberty, while other family members carrying only 1 mutant allele display a normal reproductive phenotype.

**Discussion**

IHH appears to follow the pattern of several disorders that were initially thought to be monogenic but have subsequently proven to be caused by or modulated by more than 1 gene defect (20, 21). In contrast to polygenic traits, these oligogenic disorders involve the synergistic action of mutant alleles at a small number of loci. This report expands our understanding of the genetics of IHH and



**Figure 5**

The R470L *FGFR1* mutation is loss-of-function. (A) The R→L substitution abolishes hydrogen bonds that play a role in the positioning of the C-helix of *FGFR1*. The unphosphorylated “low-activity” form of the *FGFR1* kinase domain (PDB ID: 1FGK) is represented as a ribbon diagram. The N-lobe of the *FGFR1* kinase is shown in green, with the exception of the  $\alpha$ C helix, which is in blue. Red: C-lobe of *FGFR1* kinase; gray: linker connecting the N- and C-lobes; yellow: activation loop in the C-lobe. Selected residues are shown, and hydrogen bonds are represented as dashed lines. R470 indirectly contributes to the proper positioning of  $\alpha$ C in the kinase domain and hence kinase regulation by engaging in 3 hydrogen bonds with D468, which in turn engages in a hydrogen bond with K536 from  $\alpha$ C helix. (B) The R470L mutation reduces the tyrosine kinase activity of *FGFR1*.

suggests oligogenicity may underly other monogenic disorders also characterized by incomplete penetrance and variable phenotypes within and between families.

IHH is currently described as a monogenic disorder resulting in defective GnRH secretion or action, with 7 loci implicated in the pathogenesis of the disease to date. However, the genotype-phenotype correlations from specific mutations in different loci have been imprecise. An illustrative example is *FGFR1*. Numerous heterozygous *FGFR1* mutations underlie cases of KS (4, 13, 22–25) and nIHH (7, 26), characterized by marked phenotypic variability both within and between families and apparent incomplete penetrance. Furthermore, although *FGFR1* mutations are known as causing an autosomal dominant form of IHH, there is a report of 1 KS subject with a very severe phenotype harboring a homozygous *FGFR1* mutation (4). These data suggest a dosage effect of the *FGFR1* mutant alleles and the existence of modifier genes and/or environmental effects leading to phenotypic variability across and within families.

Herein, we report a proband who carries heterozygous mutations in both *FGFR1* and *NELF* and exhibits a severe KS phenotype with cleft palate (pedigree 1). The *FGFR1* mutation, L342S, alters the spectrum of FGF binding by decreasing FGF8b binding specifically. These results were corroborated by our reporter gene assay, which showed the L342S mutation to be a loss-of-function

mutation, potentially having a dominant-negative effect in the heterozygous state. In addition, these data implicate FGF8b as a key ligand for *FGFR1c* in the pathogenesis of KS. These findings are in accordance with studies on mice with a hypomorphic *Fgf8* allele, which exhibited defected nasal cavity development and olfactory bulbs dysgenesis, a phenotype similar to that observed in the *Fgfr1* conditional knockout mouse (27–29). Therefore, we believe that the L342S *FGFR1* mutation contributed to both the KS and the craniofacial phenotype of the proband. However, the KS phenotype failed to segregate with the L342S mutation, as only the proband exhibited KS. Interestingly, he carried an additional heterozygous intronic *NELF* deletion resulting in missplicing of exon 10 and a premature stop codon. *NELF* plays a key role in GnRH and olfactory neuron outgrowth (30–32) and is colocalized with GnRH1 in human olfactory stem cells (Figure 4D). *NELF* has previously been implicated as a potential locus for IHH in an affected hypogonadotropic patient with a unique heterozygous mutation (c.1438A→G; p.T480A), though the biology of this mutant remains unexplored (10). Interestingly, the mother (no. 1-02), who carried the *NELF* mutation only, had Duane ocular retraction syndrome (OMIM 126800), a disorder of neuronal migration that has been associated with KS (33). Therefore, we believe that the heterozygous mutations in both the *NELF* and the *FGFR1* gene synergized to cause severe KS in the proband, while individuals carrying only 1 of the 2 mutations do not present with KS.

Since 1997, *GNRHR* mutations have been known to cause a recessive form of nIHH. However, unexplained variable expressivity of IHH across pedigrees carrying the same compound heterozygous mutation in *GNRHR* (6, 14) led us to screen further candidate genes. As a result, we identified a triallelic mode of inheritance for IHH in pedigree 2. Subjects carrying only 1 mutant allele (*FGFR1* or *GNRHR*) had a normal reproductive phenotype. Further, the individual (no. 1-01) carrying both heterozygous *FGFR1* and *GNRHR* mutations had only a mild reproductive phenotype (delayed puberty), while the 2 sisters harboring both a loss-of-function compound heterozygous mutation in *GNRHR* and a loss-of-function heterozygous *FGFR1* mutation (R470L) had severe IHH phenotypes. Although IHH was initially thought to be transmitted as a recessive trait, this pedigree indicates a triallelic digenic inheritance of IHH, suggesting that in some families, more than 2 mutant alleles might be required to manifest IHH. Similar patterns of mutation lead to Bardet-Biedl syndrome (34).

Only 30% of IHH subjects studied to date harbor a mutation in 1 of the genes known to cause IHH, and thus it is not possible to infer the frequency of oligogenicity in IHH. The concept of digenicity was evoked but not proven in a recent report that described a KS subject carrying both a *KALI* missense mutation and a *PROKR2* missense mutation (5). However, the functionality of these mutants was not explored, and no data were presented on the pedigree of this subject.

The discovery of oligogenic traits in IHH raises both conceptual and practical issues. Because IHH is no longer considered to be a monogenic disorder, the transmission of a trait through families is no longer synonymous with the transmission of 1 specific mutant allele. Therefore, we must rethink our current approach in studying the genetics of IHH, separating the segregation of a trait from the segregation of a specific mutant allele (21). The results of this study indicate that labeling mutant alleles as dominant or recessive is often an oversimplification. Oligogenicity also has implications for genetic counseling regarding IHH. Finally, the question remains



as to whether the variability of expressivity in IHH is controlled by a small number of major loci or a large number of minor loci.

The molecular basis of oligogenicity is poorly understood. To date, one of the best examples of the molecular basis of digenic inheritance is the direct interaction of the mutants retinal outer segment membrane protein 1 and retinal degeneration slow, which causes retinitis pigmentosa (35). The 2 mutants combine to prevent the formation of a tetrameric complex that is important for the integrity of the photoreceptors. Further examples in which synergistic mutations at different loci cause a disease are found in diseases characterized by mutations in receptor-ligand pairs, such as Hirschsprung disease (RET and GDNF) (36). In addition, indirect interactions between 2 mutated proteins have been reported in severe insulin resistance where PPARG and PPP1R3A mutants are expressed in 2 different tissues (37).

In contrast, little is known about the molecular pathways whereby the 2 pairs of mutant proteins (NELF and FGFR1; GnRHR and FGFR1) contribute to the IHH phenotype. FGFR1 and NELF are both expressed in GnRH neurons (30, 38, 39). Therefore, the 2 mutant proteins may act at different levels of the same intracellular pathway and may quantitatively contribute to its progressive dysfunction until a critical threshold is reached, thus producing the disease phenotype. Alternatively, these 2 proteins may participate in a multiprotein complex that becomes progressively compromised by the additional mutations. In the other gene pair (*FGFR1* and *GNRHR*), the *FGFR1* mutant is anticipated to reduce the number of GnRH neurons, as evidenced by a mouse model with targeted dominant-negative *FGFR1* in the GnRH neurons (40). These mice, although fertile, display a 30% decrease in the number of GnRH neurons in the hypothalamus. Conversely, the *GNRHR* mutants result in GnRH resistance that would require a compensatory increase in GnRH to restore reproductive function. Such a putative compensatory mechanism would be impaired in the individuals with an additional *FGFR1* mutation. Thus, deficient GnRH migration, secretion, and/or action could be caused by the interaction of these mutants in the same or different developmental pathways, leading to subtle amplification effects of each mutant protein.

This report expands our understanding of the genetics of this disorder by demonstrating that IHH can be caused by the combination of gene defects. The complexity of this genetic model implies a revision of the genetic terminology of IHH. Furthermore, these data predict that mutational analysis in multiple genes for seemingly monogenic disorders will become increasingly frequent and may lead to greater accuracy in phenotypic predictions. In the case of IHH, studying the interaction of a small number of key loci will undoubtedly be key to understanding some of the broader variability of the timing of normal puberty.

## Methods

**Mutational analysis.** Genetic studies were approved by Partners Healthcare Human Research Committee, and all participants provided written informed consent.

Whole blood samples were obtained from subjects and genomic DNA extracted. Exons and exon-intron boundaries were amplified using standard PCR techniques for *GNRHR* (Genbank accession number NM\_001012763 [ref. 41]); *KALI* (accession number NM\_000216 [ref. 42]); *GPR54* (accession number NM\_032551 [ref. 9]); *FGFR1* (accession number BC018128 [ref. 4]); and *NELF* (accession number ENSG00000165802 [ref. 10]). Nonsense changes resulting in a truncated protein, frameshift,

insertion, or deletion were categorized as definite mutations. Nucleotide changes, which were (a) absent from the Single Nucleotide Polymorphism database (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) and expressed sequence tags and (b) absent in at least 170 ethnically matched healthy controls were identified as mutations. All genes and proteins are described using standard nomenclature (43).

**Control population.** To determine whether the observed bp changes in the genes cited above were normal variants, a cohort of adult healthy white subjects ( $n \geq 200$ ) were screened.

**Structural analysis of the effects of *FGFR1* mutations on *FGFR1* function.** Crystal structures of the *FGFR1* kinase domain (Research Collaboratory for Structural Bioinformatics Protein Data Bank identification [PDB ID]: 1FGK) (19) and the extracellular ligand binding region of *FGFR2c* in complex with FGF8b and heparin oligosaccharide (PDB ID: 2FDB) (18) were used to map the potential effects of the mutations. Crystal structures were visualized using program O (44).

**SPR analysis.** Real-time biomolecular interactions between WT and L342S mutant *FGFR1c* extracellular domains with FGF1, FGF2, and FGF8 were characterized with a Biacore 3000 instrument as previously described (18).

**Plasmids and *in vitro* mutagenesis.** Human WT *FGFR1c* cDNA (NM\_000604) was subcloned into a pcDNA3.1<sup>+</sup> expression vector (Invitrogen). The *FGFR1c* L342S mutation was introduced into the human *FGFR1c* cDNA by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit; Stratagene) and subcloned into pcDNA3.1<sup>+</sup> using HindIII and XhoI restriction sites.

**Reporter gene assay.** To demonstrate that the heterozygous L342S mutation was a loss-of-function mutation, we used an FGF reporter bioassay. WT *FGFR1c* and L342S *FGFR1c* were transfected along with an osteocalcin FGF response element (OCFRE) promoter/luciferase reporter (45) into L6 myoblasts, a cell line largely devoid of endogenous FGFRs and FGFs (46, 47). L6 myoblasts were maintained in DMEM containing penicillin (100 U/l), streptomycin (100 µg/l), and 10% FCS (Invitrogen). A total of  $4 \times 10^4$  cells per well in 24-well plates were seeded and 24 hours later transiently transfected with a total of 400 ng of DNA consisting of 100 ng of WT *FGFR1c* cDNA, 30 ng of OCFRE reporter gene, and 0, 100, and 200 ng of the mutated *FGFR1c* (L342S) and empty vector using FuGENE 6 Reagent (Roche Diagnostics). Twenty-four hours after transfection, the cells were treated with increasing doses of FGF8b (from 0 to 2,000 pM) in culture medium containing 0.1% FCS and 1 µg/ml heparin. Following 16 hours of incubation, the cells were lysed, and luciferase activities were measured using Promega Luciferase Assay System (Promega). Since *Renilla* and  $\beta$ -galactosidase were induced by FGFs, the luciferase data are reported directly. Experiments were performed in triplicate and repeated once. The data are reported as mean  $\pm$  SD of a representative experiment performed in triplicate.

**Kinase assay.** The tyrosine autophosphorylation activity of the WT and R470L mutant *FGFR1* kinase domains were quantified using a continuous spectrophotometric assay as previously described (48).

**Generation of the *NELF* mutant construct.** To assess the effect of the intronic deletion of the *NELF* gene on exon splicing, WT and mutant genomic *NELF* constructs were made in the expression vector pCR3.1 and transfected into HEK-293 cells (Lipofectamine 2000; Invitrogen). Oligonucleotide primers (5': CAGTGACCTGCAGAGCTC-3' and 5': CCAGATCTTGGCTCCCTTGTG-3') spanning the genomic sequence from within exons 8 and 11 were used to amplify, via PCR, a fragment of WT DNA (794 bp) and mutant DNA (786 bp in the deleted allele) (Figure 4B). RT-PCR was expected to yield a 291-bp product from a *NELF* cDNA with correct splicing of exons 9 and 10.

**Immunohistochemistry of FNC-B4 cells.** FNC-B4 cells, isolated from human fetal olfactory epithelia, have characteristics of both secretory and migratory GnRH neurons (39, 49). FNC-B4 cells were stained with either GnRH1 or *NELF* antibodies (30, 49). Secondary antibodies conjugated with either Texas red or FITC were used to visualize GnRH1 and *NELF* staining.



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