Kiyonori Miura · James S. Acierno Jr.
Stephanie B. Seminara

Characterization of the human nasal embryonic LHRH factor gene, *NELF*, and a mutation screening among 65 patients with idiopathic hypogonadotropic hypogonadism (IHH)

Received: 18 December 2003 / Accepted: 9 February 2004 / Published online: 15 April 2004
© The Japan Society of Human Genetics and Springer-Verlag 2004

Abstract As the mouse nasal embryonic LHRH factor gene (*Nelf*) encodes a guidance molecule for the migration of the olfactory axon and gonadotropin-releasing hormone neurons, its human homolog, *NELF*, is a candidate gene for Kallmann syndrome, a disease of idiopathic hypogonadotropic hypogonadism (IHH) with anosmia or hyposmia. We report here characterization of *NELF* and results of mutation analysis in 65 IHH patients. Assembling EST clones, RACE, and sequencing showed that *NELF* mapped to 9q34.3 is composed of 16 exons and 15 introns with a 1,590-bp ORF encoding 530 amino acids. RT-PCR on a fetal brain cDNA library revealed five alternatively spliced variants. Among them, *NELF-v1* has 93–94% identity at the amino acid level to mouse/rat *Nelf*, and four other transcripts are also highly conserved among the three species. A 3.0-kb transcript is expressed most highly in the adult and fetal brain, testis, and kidney, indicating that *NELF* plays a role in the function of these tissues. Mutation screening detected in a patient with IHH one novel heterozygous missense mutation (1438A>G, T480A) at the donor-splice site in exon 15 of *NELF*. As this mutation was not found in 100 normal control individuals, T480A may be associated with IHH. Four other novel SNPs (102C>T and 1029C>T within the coding region, and two IVS14+47C>T and IVS15+41G>A) were also identified in *NELF*.

Keywords Human nasal embryonic LHRH factor gene · GnRH cell migration · Alternatively spliced variants · Expression pattern · Polymorphism

Introduction

Gonadotropin-releasing hormone (GnRH) is essential for the neuroendocrine control of reproduction. Previous studies of mouse embryos demonstrated that GnRH cells originate in the olfactory placode and then migrate with the olfactory nerves to reach their final destination in the hypothalamus (Wray et al. 1989; Schwanzel-Fukuda et al. 1992; Wray 2002). In the human, migrating GnRH-containing neurons play a critical role in the pathogenesis of Kallmann syndrome (KS), a disease with idiopathic hypogonadotropic hypogonadism (IHH) and anosmia/hyposmia (Waldstreicher et al. 1996; Seminara et al. 1998). One-third of IHH patients were familial, while the remaining patients were sporadic, and autosomal dominant, autosomal recessive, and X-linked forms of IHH have been reported (Waldstreicher et al. 1996). Protein of *KALI*, the gene responsible for the X-linked KS (Legouis et al. 1991; Nagata et al. 2000), exhibits characteristics as a cell adhesion molecule and is involved in both migration of GnRH cells and olfactory neuronal cells. Failure of GnRH-cell migration has been confirmed in a KS fetus (Schwanzel-Fukuda et al. 1989; Hardelin 2001), and mutation in *KALI*, *DAX*, and *LHRHR* account for ~20% of patients with hypogonadotropic hypogonadism (Seminara et al. 1999; Oliveira et al. 2001; Bera-nova et al. 2001). Thus, KS patients might also be caused by other genes.

Recently, a novel mouse protein, Nelf (nasal embryonic LHRH factor), was isolated (Kramer and Wray 2000), and its gene (*Nelf*) is expressed in the olfactory sensory cells and GnRH cells during embryonic development. Nelf serves as a common guidance molecule for the olfactory axon and GnRH neurons across the nasal

K. Miura · J. S. Acierno Jr. · S. B. Seminara
Reproductive Endocrine Unit,
Massachusetts General Hospital,
Boston, MA 02114, USA

Present address: K. Miura (✉)
Department of Obstetrics and Gynecology,
Nagasaki University Graduate School of Biomedical Sciences,
1-7-1 Sakamoto, Nagasaki 852-8501, Japan
E-mail: kiyonori@net.nagasaki-u.ac.jp
Tel.: +81-95-849-7363
Fax: +81-95-849-7365

region (Kramer and Wray 2000). In addition, *Nelf* knocked-down mice inhibited the olfactory axon outgrowth and abnormal migration of the GnRH cell into the periphery of nasal explant (Kramer and Wray 2000). These findings suggest that the human homolog, *NELF*, is a functional candidate gene for KS. Here we report the characterization of *NELF* and a mutation screening in patients with IHH.

Materials and methods

Human *NELF*

We characterized *NELF* by assembling human EST clones, RACE, and RT-PCR on a human fetal brain cDNA library followed by sequence comparison with the human genomic data (<http://www.ncbi.nih.gov/>). Subcloned *NELF* cDNAs were digested to generate probes (exon 3 probe) for Northern blot hybridization. Digestion with *RsaI* (5 U/ μ g DNA) created a 304-bp fragment, corresponding to positions 304–607 within exon 3 of *NELF*. Radiolabeled exon 3 probe was hybridized to 2 μ g poly (A)⁺ RNA according to the

manufacturer's instruction (Human Multiple Tissue Northern Blot, Clontech).

Screening for *NELF* mutations in patients with IHH

Sixty-five patients with IHH (33 patients with KS) who lacked *KALI* mutations were screened for mutations in *NELF*. Of the 65 patients, 35 (21 of 33 KS patients) had a family history of IHH other than that suggesting an X-linked inheritance. All subjects met the following criteria: (1) failure to completion of puberty by the age of 18 years; (2) low serum testosterone concentration (<100 ng/dl for men and estradiol <20 pg/ml for women); (3) low or normal levels of circulating gonadotropins (for normal values, see Waldstreicher et al. 1996; Kratz and Lewandrowski 1998); and (4) normal findings in the brain by magnetic resonance imaging. Genomic DNA was extracted from their peripheral blood leukocytes. Primers were designed to amplify each exon, including acceptor–donor splice sites, of *NELF* (Table 1). When a base-pair change in *NELF* was detected, 100 healthy control samples were examined for the change. This study was approved by the Subcom-

Table 1 PCR primers used for amplification of the coding sequence and sequence variations of human *NELF*. T_m indicates annealing temperatures for PCR conditions. Freq indicates allele frequency

Exon	Name	Sequence	Size of products (bp)	T_m (°C)	Sequence variation	Amino acid change	Allele	Freq
1	N1F	GCCCCGCCCCAACTCAACC	625	68				
	N1R	TAGCAGGGCAGGGGACCAGGAAGA						
2	N2F	GGCCAGGGCGCACCAGGACCAA	390	65	102C > T	S34S	T	0.33
	N2R	CGGGCAGGGAGGGCAGGGGATGTA					C	0.67
3	N3F	ACTGGGTGTTCTCTGCCTTC	622	65				
	N3R	AGCGGCATGCACCCCCAAACCT						
4	N4F	CTGGACCCAGAAGCATCTG	304	65				
	N4R	TGGCACAGCATAGGCACG						
5	N5F	GACATCAGCTTCCGTTGCCATTC	406	68				
	N5R	AGTCCCAGCCTGCCTCACCT						
6	N6F	GGGCCCTCTGCTCGCACCAGTGC	262	68				
	N6R	GTCGCCACCACCCTTCCCCCATCA						
7	N7F	CTGGAAGGCTGTCGCGGGAAGAG	351	65				
	N7R	ATAGGGGTTGGGGCGGGGGTCGTT						
8	N8F	GTCTGCTTCATGTGCCTTAG	394	60				
	N8R	CTTTGCGATCGGAGATGCTG						
9	N9F	GCCATGGCCAGGATAGGAGGTG	288	65	1261G > A	F343F	A	0.42
	N9R	GCTGGAGAAGGCTGCGTGGTC					G	0.58
10	N10F	AGCCAGAAGGTAGCCTCATG	293	62				
	N10R	ACATCAAGGCTGCGTCAGAGC						
11	N11F	CAACAGATGCCCTCTTGCG	268	65				
	N11R	AGAGCGGGTGTGAGTGCTG						
12	N12F	ATGAGCACGCAACCTTCGAG	264	62				
	N12R	CACGATTCTTCTCCTGGTCAG						
13	N13F	GGCCGGGGGATGAAGACCAA	303	65				
	N13R	GACAGGCACCCCACTCCACAGA						
14	N14F	CGGTCCGCTCTGTGGAGT	248	62	IVS14+47C > T	Intron14	T	0.22
	N14R	AGGAAACATGGAAGGCTCTG					C	0.78
15	N15F	CCATCTCCTCCCCGCTCCTCTTC	275	68	1438A > G	T480A	G	
	N15R	AGTACGTCTCGATCATCTGCTTCC					A	
16	N16F	TGAGCCCAGCCAGGTTT	450	65	IVS15+41G > A		A	0.37
	N16R	CCGGTGCAGAGGGAGTG					G	0.63

Fig. 1 Northern blot analysis of human *NELF*: upper pane shows the *NELF* expression, and lower pane the beta-actin expression as a control. A 4.9-kb major transcript is expressed in all tissues examined, and a 3.2-kb transcript is seen in the brain only, whereas a 3.0-kb transcript identical in size to the *NELF* consensus mRNA is highly expressed in the brain, kidney, and testis

mittee on Human Studies of the Massachusetts General Hospital, and all participants provided written informed consent.

Results and discussion

Characterization of human *NELF*

RT-PCR of *NELF* using the human fetal brain cDNA library revealed five alternatively spliced variants. *NELF-v1* (GenBank accession no. AY255128), corresponding to the full-length cDNA and composed of 16 exons and 15 introns with a 1,590-bp ORF, is predicted to encode a protein of 530 amino acids. *NELF-v2* (GenBank accession no. AY255129) lacks 6 bp comprising the entire sequence of exon 5. *NELF-v3* (GenBank accession no. AY255130) has the sequence missing 69 bp that corresponds to exon 6. *NELF-v4* (GenBank accession no. AY255131) lacks 90 bp for exon 8. *NELF-v5* (GenBank accession no. AY255132) includes 236 bp from intron 9. Size of the five transcripts excluding the poly (A) tail is 2,994, 2,988, 2,925, 2,904, and 3,230 bp, respectively. *NELF-v1* shows 93 and 94% identity at the amino acid level to mouse *Nelf* (GenBank accession no. BAC25597) and rat *Nelf* (GenBank accession no. CAC20866), respectively. All these transcripts isolated are highly conserved among the human, mouse, and rat. Using the public database (BLASTP and Swiss-prot), no genes or proteins homologous to human *NELF-v1* were identified. Although human genome browser suggests the existence of two ESTs with possible novel splice sites (GA/AG in exon 15, GenBank accession no. AI871403; and GC/GG in exon 16, GenBank accession no. AK074602), RT-PCR in the present study never detected such spliced transcripts. The result suggested their very low expression in the human fetal brain.

Multiple-tissue Northern blot analysis revealed 4.9 and 3.0-kb mRNA in all the tissues (Fig. 1). The 3.0-kb transcript with the highest expression level appeared in the adult and fetal brain, testis (fetal ovary not tested), and kidney, indicating that *NELF* may play an important role in the function of these tissues. In the 3' RACE and database search, we found only one poly (A) signal (GenBank accession no. AY255128) located 1,147 bp

Fig. 2a,b Analysis for 1438A>G substitution. **a** An *ApaI* site created by the mutation. **b** Electropherogram of *ApaI* fragments from sibs (lanes 1 and 2), the patient (lane 3), a control (lane 4), and size marker (lane 5)

downstream to the stop codon and generating the 3.0-kb transcript. However, the usage of either another poly (A) signal located in the gap sequence downstream to *NELF* or a >2.0-kb poly (A) tail cannot be excluded. This may lead to the 4.9-kb transcript. The arguments are supported by the fact that the mouse *Nelf* uses two different poly (A) signals generating 2,924-bp and 4,607-bp mRNA, respectively. An additional 3.2-kb transcript from human *NELF* was also found in the adult and fetal brain, raising the possibility of a tissue-specific splice variant.

Mutation analysis

We detected one heterozygous missense mutation (1438A>G, T480A) at the donor-splice site in exon 15 of *NELF* (GenBank accession no. AI871403) in a sporadic case of IHH (Fig. 2). This amino acid substitution switches from a polar side chain in threonine to a non-polar side chain in alanine. Neither the 100 normal control individuals had an *ApaI* site that is newly created

by the A → G change, nor was any such substitution found in the public SNP database. These findings, together with the fact that Thr480 is highly conserved among the mouse, rat, and human, suggest that T480A is associated with the pathogenesis of IHH. Alternatively, the 1438A > G substitution may be a rare polymorphism (SNP). However, as samples from the patient were no longer available, it remains to be seen whether the mutation leads to dysfunction of the gene. Four novel base substitutions (102C > T and 1029C > T within the coding region and IVS14+47C > T and IVS15+41G > A) were also identified in *NELF* (Table 1). They will be useful in further studies to determine a possible relationship between *NELF* and any reproduction and olfaction disorders.

Acknowledgement We thank Dr. William F. Crowley Jr for his help and valuable advice.

References

- Beranova M, Oliveira LM, Bedecarrats GY, Schipani E, Vallejo M, Ammini AC, Quintos JB, Hall JE, Martin KA, Hayes FJ, Pitteloud N, Kaiser UB, Crowley WF Jr, Seminara SB (2001) Prevalence, phenotypic spectrum, and modes of inheritance of gonadotropin-releasing hormone receptor mutations in idiopathic hypogonadotropic hypogonadism. *J Clin Endocrinol Metab* 86:1580–1588
- Hardelin JP (2001) Kallmann syndrome: towards molecular pathogenesis. *Mol Cell Endocrinol* 179:75–81
- Kramer PR, Wray S (2000) Novel gene expressed in nasal region influences outgrowth of olfactory axons and migration of luteinizing hormone-releasing hormone (LHRH) neurons. *Genes Dev* 14:1824–1834
- Kratz A, Lewandrowski KB (1998) Normal reference laboratory values. *N Engl J Med* 339:1063–1072
- Legouis R, Hardelin JP, Claverie JM, Compain S, Wunderle V, Millasseau P (1991) The candidate gene for the X-linked Kallmann syndrome encodes a protein related to adhesion molecules. *Cell* 67:423–435
- Nagata K, Yamamoto T, Chikumi H, Ikeda T, Yamamoto H, Hashimoto K, Yoneda K, Nanba E, Ninomiya H, Ishitobi K (2000) A novel interstitial deletion of KAL1 in a Japanese family with Kallmann syndrome. *J Hum Genet* 45:237–240
- Oliveira LM, Seminara SB, Beranova M, Hayes FJ, Valkenburgh SB, Schipani E, Costa EM, Latronico AC, Crowley WF Jr, Vallejo M (2001) The importance of autosomal genes in Kallmann syndrome: genotype–phenotype correlations and neuroendocrine characteristics. *J Clin Endocrinol Metab* 86:1532–1538
- Schwanzel-Fukuda M, Bick D, Pfaff DW (1989) Luteinizing hormone-releasing hormone (LH-RH) expressing cells do not migrate normally in an inherited hypogonadal (Kallmann) syndrome. *Mol Brain Res* 6:311–326
- Schwanzel-Fukuda M, Jorgenson KL, Bergen HT, Weesner GD, Pfaff DW (1992) Biology of normal luteinizing hormone-releasing hormone neurons during and after their migration from olfactory placode. *Endocr Rev* 13:623–634
- Seminara SB, Hayes FJ, Crowley WF Jr (1998) Gonadotropin-releasing hormone deficiency in the human (idiopathic hypogonadotropic hypogonadism and Kallmann's syndrome): pathophysiological and genetic considerations. *Endocr Rev* 19:521–539
- Seminara SB, Achermann JC, Genel M, Jameson JL, Crowley WF Jr (1999) X-linked adrenal hypoplasia congenita: a mutation in DAX1 expands the phenotypic spectrum in males and females. *J Clin Endocrinol Metab* 84:4501–4509
- Waldstreicher J, Seminara SB, Jameson JL, Geyer A, Nachtigall LB, Boepple PA, Holmes LB, Crowley WF Jr (1996) The genetic and clinical heterogeneity of gonadotropin-releasing hormone deficiency in the human. *J Clin Endocrinol Metab* 81:4388–4395
- Wray S (2002) Molecular mechanisms for migration of placodally derived GnRH neurons. *Chem Senses* 27:569–572
- Wray S, Grant P, Gainer H (1989) Evidence that cells expressing luteinizing hormone-releasing hormone mRNA in the mouse are derived from progenitor cells in the olfactory placode. *Proc Natl Acad Sci USA* 86:8132–8136