CLINICAL STUDY

Mutational analysis of the necdin gene in patients with congenital isolated hypogonadotropic hypogonadism

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Abstract

Context: Necdin activates GNRH gene expression and is fundamental for the development, migration, and axonal extension of murine GNRH neurons. In humans, necdin plays a potential role in the hypogonadotropic hypogonadism phenotype in patients with Prader–Willi syndrome.

Aim: To investigate necdin gene (NDN) variants in patients with isolated hypogonadotropic hypogonadism (IHH).

Patients and methods: We studied 160 Brazilian patients with IHH, which includes 92 with Kallmann syndrome and 68 with normosmic IHH. Genomic DNA was extracted and the single NDN exon was amplified and sequenced. To measure GNRH transcriptional activity, luciferase reporter plasmids containing GNRH regulatory regions were transiently transfected into GT1-7 cells in the presence and absence of overexpressed wild-type or mutant necdin.

Results: A heterozygous variant of necdin, p.V318A, was identified in a 23-year-old male with Kallmann syndrome. The p.V318A was also present in affected aunt and his father and was absent in 100 Brazilian control subjects. Previous FGFR1 gene analysis revealed a missense mutation (p.P366L) in this family. Functional studies revealed a minor difference in the activation of GNRH transcription by mutant protein compared with wild type in that a significant impairment of the necdin protein activity threshold was observed.

Conclusion: A rare variant of necdin (p.V318A) was described in a family with Kallmann syndrome associated with a FGFR1 mutation. Familial segregation and in vitro analysis suggested that this non-synonymous variant did not have a direct causative role in the hypogonadism phenotype. NDN mutations are not a frequent cause of congenital IHH.

Introduction

Congenital isolated hypogonadotropic hypogonadism (IHH) is characterized by complete or partial failure of pubertal development due to impaired secretion of LH and FSH in the absence of lesions of the CN or other organic cause (1, 2). When IHH is associated with abnormal olfactory function (anosmia or hyposmia), it is defined as Kallmann syndrome (1). IHH is a genetically heterogeneous disorder, which can be inherited as an autosomal dominant, recessive, or X-linked trait (1).

New promising candidate loci for IHH include genes with potential influence on GNRH neuron migration, secretion, or action (3). In addition, clues to determine candidate genes for GNRH deficiency in humans could also be gleaned by other syndromes that overlap the hypogonadism and olfactory abnormalities, as exemplified by CHD7 gene mutations, responsible for CHARGE syndrome, recently associated with IHH (4). Prader–Willi syndrome (PWS) is a contiguous multigene disorder characterized by hyperphagia, obesity, and isolated IHH, all highly suggestive of hypothalamic dysfunction (5). Interestingly, recent studies demonstrated that a primary testicular defect can also contribute to the hypogonadism in PWS males (6). PWS arises from the lack of expression of paternally inherited imprinted genes on chromosome 15q11-q13, including the necdin gene (NDN), and possibly other genes within this chromosome region, due to the paternal intra-chromosome deletion or maternal uniparental disomy (5).

Necdin belongs to the MAGE superfamily of proteins and plays critical roles in neuronal differentiation (7). It has recently been shown to be a key regulator of GNRH gene expression and neuronal development in rodents (7, 8). The necdin-null mouse exhibited a decreased number of GNRH neurons and impaired...
GNRH neuronal migration during development (9). These findings suggest that a loss of necdin function may contribute to hypogonadotropic hypogonadism and infertility phenotypes in PWS (8).

We hypothesized that NDN might be implicated in the pathogenesis of congenital IHH, without other signs of PWS in humans. NDN gene defects were investigated in a cohort of Brazilian patients with isolated GNRH deficiency, including Kallmann syndrome and normosmic IHH.

**Materials and methods**

**Patients**

A total of 160 Brazilian patients with sporadic or familial IHH were studied, which includes 92 patients with Kallmann syndrome (77 males and 15 females) and 68 patients with normosmic IHH (50 males and 18 females). Informed written consent was obtained from all patients, and the study was approved by the ethics committee of the Hospital das Clinicas, Sao Paulo University. KAL1 and FGFR1 had previously been studied in the cohort with Kallmann syndrome and GNRHR, FGFR1, KISS1R, KISS1, TAC3, and TAC3R had been studied in the normosmic IHH patients (10). The control population composed of 100 healthy Brazilian adults of both sexes with a history of normal pubertal development.

Idiopathic IHH was diagnosed based on the following criteria: incomplete or absent pubertal development after 18 years, prepubertal or low testosterone or estradiol levels for age, and low or normal basal gonadotropin levels but otherwise normal pituitary function, and normal hypothalamic–pituitary imaging. Olfactory tests (Smell Identification Test, Philadelphia, PA, USA or Alcohol Sniff Test, San Diego, CA, USA) were performed in the majority of the patients with IHH. (11).

**DNA analysis**

Genomic DNA was extracted from peripheral blood leukocytes using standard procedures. The single exon of the human NDN (GenBank accession number – MIM602117) was amplified using the following primer pairs: N1 – forward 5'-CTTAAAAGTGGCCCTTTCTC-3' and reverse 5'-CATGATTTGCATCTTGGTG-3'; and N2 – forward 5'-AAGAAGTGGTGGCAGGAGCAT-3' and reverse 5'-GGGATTTTTCCCCACCTATT-3'. Amplification reactions were performed in a final volume of 25 µl containing 200 ng genomic DNA, 0.2 mM dNTPs, 1.5 mM enhancer, 0.6 pmol each primer, 1× PCR buffer, and 1 U Go Taq DNA polymerase (Promega) and carried out for 35 cycles: denaturation at 95 °C for 30 s, annealing at 52–56 °C for 30 s, extension at 72 °C for 1 min, followed by a final extension for 10 min at 72 °C. The PCR products were examined on 1% agarose gel electrophoresis and sequenced in an ABI Prism Genetic Analyzer 3100 automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA).

**Plasmids**

pGL3-basic luciferase reporter plasmids (Promega) containing GNRH regulatory elements GNRH enhancer-1/GNRH promoter (GNRH-E1/GNRH-P), −2980/−2631-GNRH-E1/GNRH-P, GNRH-E2/GNRH-E1/GNRH-P, and GNRH-E3/GNRH-E2/GNRH-E1/GNRH-P have previously been described (12–14). pcDNA4/HisMaxC empty expression plasmid and pcDNA4/HisMaxC-necdin containing the coding sequence for mouse Ndn have been described (8). The mutant Ndn p.V322A (homologous to human NDN p.V318A) was created in pcDNA4/HisMaxC-necdin using the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA, USA).

**Cell culture, transfections, and reporter assays**

GT1-7 mature GNRH neuronal cells (15) were cultured in DMEM with 4.5% glucose, 10% fetal bovine serum, and 1× penicillin–streptomycin in 5% CO₂ at 37 °C. Cells were seeded into 24-well plates at 90 000 cells per well for ~24 h before transfection. Transient transfections were performed using FuGENE 6 Transfection Reagent (Roche Applied Science) according to the manufacturer’s recommendations. Cells were co-transfected with 400 ng/well luciferase reporter plasmid and 50, 75, 100, or 200 ng/well expression plasmid containing wild-type or p.V322A Ndn as well as 100 ng/well thymidine kinase-β-galactosidase reporter plasmid as an internal control for transfection efficiency. Cells were harvested 48 h post-transfection in lysis buffer (100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100). Luciferase assays were performed as described previously (16) and β-galactosidase assays were performed using the Galacto-Light Plus assay system as directed by the manufacturer (Applied Biosystems). Luciferase values were normalized to β-galactosidase values to control for transfection efficiency. Data are presented as fold luciferase/β-galactosidase activity, relative to empty expression vector control, mean ± s.d., of four independent experiments. Statistical analyses were performed by Student’s t-test with P<0.05 to indicate significance.

**Results**

**DNA analysis of the NDN gene**

Sequencing of the NDN gene revealed a heterozygous T to C transition in coding nucleotide 953 (c.953T>C; Fig. 1A), in a male with familial Kallmann syndrome.
and FSH!

basal prepubertal gonadotropin levels, LH!

formal olfactory test. Hormonal evaluation revealed

complained of anosmia, which was confirmed by a

micropenis, and unilateral cryptorchidism (left). He

delayed pubertal development, scarce pubic hair,

Brazilian adults.

absent in the control population of 100 healthy

terminal region of necdin. The p.V318A variant was

alanine at position 318 (p.Val318Ala) in the carboxyl

This mutation resulted in the substitution of valine for

alanine at position 318 (p.Val318Ala) in the carboxyl

terminal region of necdin. The p.V318A variant was

absent in the control population of 100 healthy

Brazilian adults.

The proband presented at 23 years of age with

delayed pubertal development, scarce pubic hair,

micropenis, and unilateral cryptorchidism (left). He

complained of anosmia, which was confirmed by a

formal olfactory test. Hormonal evaluation revealed

basal prepubertal gonadotropin levels, LH <0.6 IU/l

and FSH <1.0 IU/l (IFMA) and low testosterone levels,

50 ng/dl (FIA). Other pituitary hormones were normal.

He was under psychiatric evaluation due to episodic

attacks of aberrant behavior, hypersomnia, megaphagia,

and increased sex drive. Familial segregation revealed

that this non-synonymous variant was present in

the homozygous state in one paternal aunt with

Kallmann syndrome and in his father who had a history

of delayed pubertal onset (Fig. 1B). The proband, as well

as his two affected aunts and his father, also carried a

previously described heterozygous FGFR1 mutation

(p.P366L) (17).

In addition, two previously known silent NDN

polymorphisms, c.11C>T (rs3743340) and c.971C>T

(rs2192206), were identified in 80 and 35 patients with

IHH respectively.

Functional analysis of the p.V318A variant

Human and mouse necdin proteins contain 321 and

325 amino acids respectively. The highly conserved

MAGE homology domain (amino acids 105–275 in

human and 109–279 in mouse) has been shown to be

critical for protein–protein interactions with various

factors such as p53 and Nogo-A, as well as for

subcellular localization (18, 19). The carboxyl terminal

region (275–321 in human and 280–321 in mouse) is

highly conserved among mammalian species but does

not yet have a characterized function. In particular, the

valine residue that is mutated in the proband with

Kallmann syndrome is 100% conserved among human,

chimpanzee, dog, cow, and rodents, which suggest an

important functional role (Fig. 1C).

To test the effect of the p.V318A variant on

necdin-mediated activation of GNRH transcription,

the homologous mutation (p.V322A) was created in

an expression plasmid containing mouse Ndn. To

measure GNRH transcriptional activity, luciferase

reporter plasmids containing various portions of the

rat GNRH regulatory regions were transiently trans-

ferred into GT1-7 cells, a model of the mature,
differentiated GNRH neuron, in the presence and

absence of overexpressed wild-type or p.V322A mutant

Ndn, similar to previous studies (8). The rat GNRH

regulatory regions are highly conserved (~80%) from

rodents to human (14). Overexpression of wild-type

Ndn resulted in a significant 50% increase in GNRH

transcriptional activity on all GNRH reporters tested.

The p.V322A Ndn significantly activated expression of

GNRH regulatory elements to an equivalent degree as

wild-type Ndn, indicating that the patient mutation did

not significantly impair necdin function (Fig. 2A).

Furthermore, p.V322A Ndn likely did not function as a

dominant negative, as it still activated GNRH

expression rather than interfered with the function of

endogenous NDN in GT1-7 cells.

To determine whether this variant in necdin

interfered with protein activity thresholds, expression

of the GNRH-E1/GNRH-P reporter was compared in

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In this study, we identified a heterozygous necdin variant (p.V318A) in a male patient with familial Kallmann syndrome. This variant was also identified in the patient’s father and in one of the proband’s affected aunts with Kallmann syndrome, both in homozygous state (Fig. 1). The p.V318A variant was absent in 200 alleles from the control individuals, suggesting that it is not a common polymorphism in the Brazilian population. The p.V318A variant was also recently identified as a rare variant in <1% of the population in the 1000 genome pilot project, which provided a deep characterization of human genome sequence variation using different genome-wide sequencing with high-throughput platforms (20).

Previous studies conducted by Trarbach et al. (17) showed that this patient and his two affected aunts also carried a heterozygous variant of the FGFR1 gene (p.P366L). In fact, IHH appears to follow the pattern of several disorders that were initially thought to be monogenic but have subsequently been found to be caused or modulated by more than one gene defect (2, 10, 21, 22). Defects in two or more genes have been observed in IHH cases, with mutations in FGFR1 associated with GNRHR, NELF, or FGFR8 and in PROKR2 associated with KAL1, PROK2, or GNRH1, partially explaining the wide phenotypical variability observed within and across families with Kallmann syndrome and normosmic IHH (2, 10, 21, 22). Though a direct causal role for necdin was not identified in this study, we observed a possible impairment of the protein activity threshold with the p.V318A necdin, due to highly variable activity at lower concentrations. Though the GNRH regulatory elements and C-terminal region of necdin are highly conserved between rodents and human, it is possible that the human protein and GNRH regulatory elements might show effects different than those observed in this study. Necdin may have additional unknown cellular functions in GNRH neuronal development that may be affected by this mutant and remains an interesting candidate for IHH. Further studies are needed to delineate the actual contribution of this gene in human pubertal development and reproduction.

GNRH1, a fundamental regulator of human reproduction, has extensively been studied by several groups in IHH patients for more than three decades, but only in 2009, GNRH1 mutations were found to be associated with IHH phenotypes (23). Indeed, studying the biological mechanisms controlling developmental GNRH gene expression and migration of GNRH neurons by NDN into the developing CN reveals additional genetic pathways that play a role in the pathogenesis of IHH. Miller et al. (8) described that necdin activation of GNRH transcription was dependent on the known Msx/Dlx-binding sites in the GNRH-E1 and promoter regions, indicating that necdin may not directly bind to the GNRH regulatory sequence but instead function as a cofactor. This finding could indicate a second pathway

Discussion

Necdin has a defined role in the migration and/or differentiation of GNRH neurons (7), suggesting that loss of necdin expression may be at least partially responsible for the hypogonadotropic hypogonadism phenotype in PWS patients, therefore making NDN a plausible candidate gene for IHH in humans.

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possibly related to regulation of GNRH neurons, the Dlx homeodomain transcriptional activators. In fact, Givens et al. (16) showed that mouse embryos null for Dlx1 and Dlx2 have 30–35% fewer GNRH neurons. Thus, a possible inactivating mutation in one or more of the genes encoding Dlx proteins, perhaps in combination with necdin, could be responsible for a decrease in the number of GNRH neurons and thereby contribute to the IHH phenotype.

In conclusion, we describe a rare variant (p.V318A) of the necdin gene in a male patient with Kallmann syndrome with a previously identified FGFR1 gene mutation. Familial segregation and functional studies of the p.V318A necdin protein suggested that this NDN variant did not have a direct causative role in the hypogonadism phenotype. Therefore, this work suggests that the findings of multiple genetic alterations need to be carefully interpreted to definitely define the actual concept of digenic or oligogenic inheritance in congenital IHH.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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