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.

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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'-CTTAAACTGGCCCTTCTC-3' and reverse 5'-CATGATTTGCATCTTGGTG-3'; and N2 – forward 5'-AAGAAGTGGTGCAGGAGCAT-3' and reverse 5'-GCGATTTTCCACCTATT-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.

Figure 1 (A) Nucleotide sequence of *NDN* gene showing the wild-type and the heterozygous variant identified in the male proband with Kallmann syndrome. (B) Pedigree of the family carrying the p.V318A variant in the necdin. The proband is identified by an arrow. Squares denote male subjects and circles denote female subjects. *NDN* and *FGFR1* genotypes are shown in the family members who were studied. (C) Top, schematic diagram of necdin. Residue numbers correspond to human necdin. Bottom, amino acid sequence alignment of the necdin carboxyl terminus among mammalian species. The p.V318A patient mutation is indicated by an arrow.

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 transfected 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

Figure 2 Effects of wild-type or variant (p.V322A) mouse *Ndn* on *GNRH* expression. (A) GT1-7 mature GNRH neuronal cells were transiently co-transfected with 400 ng of reporter plasmids containing GNRH regulatory elements driving luciferase, as indicated by schematic diagrams, with 200 ng of either empty expression vector, wild-type *Ndn* expression plasmid, or p.V322A *Ndn* expression plasmid. GT1-7 cells were transiently transfected with 400 ng GNRH-E1/GNRH-P luciferase reporter plasmid with increasing amounts (50, 75, 100, and 200 ng) of either wild-type *Ndn* or variant p.V322A *Ndn* expression plasmid. Luciferase values were normalized to a co-transfected β -galactosidase reporter to control for transfection efficiency. Data are presented as fold luciferase/ β -galactosidase activity, relative to empty expression vector control, mean $\pm$ s.d., of four independent experiments. *Significantly different from empty vector by Student's *t*-test ($P < 0.05$). E1, GNRH enhancer-1; E2, GNRH enhancer-2; E3, GNRH enhancer-3; GNRH-P, GNRH promoter.

the presence of increasing amounts of overexpressed wild-type or p.V322A necdin in GT1-7 cells. Wild-type *Ndn* significantly activated GNRH-E1/GNRH-P expression relative to empty vector in the presence of 75, 100, and 200 ng expression plasmid. p.V322A *Ndn* showed highly variable activity and only significantly activated GNRH expression relative to empty vector with the maximal 200 ng dose of expression plasmid, suggesting a potential impairment of the necdin protein activity threshold (Fig. 2B).

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.

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 *GNRHHR*, *NELF*, or *FGF8* 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

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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