RESEARCH

Homozygous *CDH2* variant may be associated with hypopituitarism without neurological disorders

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Abstract

Context: Congenital hypopituitarism is a genetically heterogeneous condition. Whole exome sequencing (WES) is a promising approach for molecular diagnosis of patients with this condition.
Objectives: The aim of this study is to conduct WES in a patient with congenital hypopituitarism born to consanguineous parents, *CDH2* screening in a cohort of patients with congenital hypopituitarism, and functional testing of a novel *CDH2* variant.
Design: Genomic DNA from a proband and her consanguineous parents was analyzed by WES. Copy number variants were evaluated. The genetic variants were filtered for population frequency (ExAC, 1000 genomes, gnomAD, and ABraOM), *in silico* prediction of pathogenicity, and gene expression in the pituitary and/or hypothalamus. Genomic DNA from 145 patients was screened for *CDH2* by Sanger sequencing.
Results: One female patient with deficiencies in growth hormone, thyroid-stimulating hormone, adrenocorticotropic hormone, luteinizing hormone, and follicle-stimulating hormone and ectopic posterior pituitary gland contained a rare homozygous c.865G>A (p.Val289Ile) variant in *CDH2.* To determine whether the p.Val289Ile variant in *CDH2* affects cell adhesion properties, we stably transfected L1 fibroblast lines, labeled the cells with lipophilic dyes, and quantified aggregation. Large aggregates formed in cells expressing wildtype *CDH2*, but aggregation was impaired in cells transfected with variant *CDH2* or non-transfected.
Conclusion: A homozygous *CDH2* allelic variant was found in one hypopituitarism patient, and the variant impaired cell aggregation function *in vitro*. No disease-causing variants were found in 145 other patients screened for *CDH2* variants. Thus, *CDH2* is a candidate gene for hypopituitarism that needs to be tested in different populations.

Key Words
- whole exome sequencing
- cell adhesion
- growth insufficiency
- CDH2

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Significance statement: A female patient with hypopituitarism was born from consanguineous parents and had a homozygous, likely pathogenic, *CDH2* variant that impairs cell aggregation *in vitro*. No other likely pathogenic variants in *CDH2* were identified in 145 hypopituitarism patients.

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Introduction

Pituitary organogenesis results from a complex interaction of signaling pathways, leading to the coordinated expression of transcription factors in a spatially and temporally regulated manner, and an appropriate balance between cell proliferation and differentiation (1). Defects in the genes that control these processes can cause congenital hypopituitarism, which is defined as the deficiency of one or more pituitary hormones.

Congenital growth hormone deficiency (GHD) occurs in approximately 1/8000 children (https://medlineplus.gov/genetics/). The frequency of mutations in the most frequently screened genes, *PROP1, POU1F1, HESX1, LHX3,* and *LHX4*, was 12.4% in a review of 21 studies (2). The mutation discovery rate was lower in sporadic cases, 11.2%, than in familial cases, 63%. Congenital hypopituitarism is genetically very heterogeneous, and by 2016, mutations in about 30 different genes were attributed to this disorder (3). Three of these genes *ARNT2, IGSF1,* and *KCNQ1* were discovered in familial cases or probands from consanguineous parents by application of whole exome sequencing (WES) technology (4, 5, 6). Since then, the broad application of WES and detection of copy number variation has implicated 37 additional genes in this disorder, with varying degrees of certainty regarding pathogenicity, in some cases. Thus, WES is a promising approach for novel gene discovery and molecular diagnosis of patients with isolated GHD and combined pituitary hormone deficiency (CPHD).

Some genes that can cause hypopituitarism are associated with additional clinically relevant effects on craniofacial development, including septo-optic dysplasia or holoprosencephaly, and/or other peripheral tissues, such as the development of the genitals, skeleton, and/or kidney (3). Genotype–phenotype correlations are not always clear, making it difficult to select candidate genes for analysis and to predict the severity of clinical presentation based on DNA sequence alone. In this study, we used WES to screen a patient with congenital hypopituitarism born from a consanguineous Brazilian family. We identified a homozygous exonic variant in N-cadherin (*CDH2*) that reduces cell aggregation, suggesting that *CDH2* is a novel candidate gene for congenital hypopituitarism.

Materials and methods

Ethical procedures

All patients or their parents gave their permission to take part in the present study, which was approved by the Brazilian national ethical committee under the number CAAE 0642812.4.0000.0068

Proband

A female patient was born from consanguineous parents and presented with CPHD (growth hormone (GH), thyroid-stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), prolactin (PRL)) and ectopic posterior pituitary lobe. DNA from the patient and her parents was submitted for WES.

Cohort

One hundred and forty-five Brazilian patients with congenital hypopituitarism were analyzed. There were 87 males and 58 females, and 143 with CPHD and 2 with GHD. All patients are clinically monitored at the Developmental Endocrinology Unit of the University of São Paulo. This study was approved by the ethics committee of this institution and the consent form was signed by all patients participating in the study before the beginning of the genetic evaluation.

Patient height was measured using a millimeter precision stadiometer, and the S.D. (Z) height score was calculated using British reference standards (8). The mean height Z ± S.D. of the sample was −4 ± 1.62, consistent with the short stature required for study participation. Weight was measured on a standard digital scale, showing an average Z BMI ± S.D. of −1 ± 1.4. The degree of pubertal development was assessed according to the Tanner scale of sexual maturity rating (9, 10). The inbreeding rate was 8% and familial cases represent 25% of the sample (Table 1).

Hormonal evaluation was performed, and the diagnosis of GHD was based on low levels of insulin-like growth factor 1 and insulin-like growth factor-binding protein 3 and failure to respond normally to

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Table 1 Clinical data of the patients selected for the study.

Sex (M:F)	87:58
Age (years)	37 ± 10.7
Age at diagnosis (years)	12 ± 8.4
Z high	−4 ± 1.62
Z BMC	−1 ± 1.4
Topic PP: Ectopic PP	1:144
Hypoplastic adenohypophysis	135 (94%)
GHD:CPHD	2:143
Consanguineous cases	12 (8.39%)
Familial cases	36 (25.1%)

BMC, body mass control; CPHD, combined pituitary hormone deficiency; F, female; GHD, growth hormone deficiency; M, male; PP, posterior pituitary.

the GH stimulation test. Glucose, cortisol, GH, TSH, PRL, luteinizing hormone (LH), follicle-stimulating hormone (FSH), tri-iodothyronine, thyroxine (T_4), free T_4, dehydroepiandrosterone sulfate, estradiol, or testosterone were measured at baseline as previously described (7). Clonidine and combined pituitary stimulation tests (0.05–0.1 U/kg insulin, 200 μg thyrotropin-releasing hormone (TRH) and 100 μg gonadotropin-releasing hormone (GnRH), iv) were performed, and GHD was diagnosed when peak GH was <7 μg/L measured by immunoradiometric assay or <3.3 μg/L measured by immunofluorescence assay (7).

The hypothalamic–pituitary region was assessed by magnetic resonance imaging using a 1.5 Tesla Signa GE device (Milwaukee, Wisconsin, USA) to obtain T1 and T2 coronal and sagittal planes with a repetition time of 350 ms and echo time of 20 ms Coronal images were obtained using 3 mm slices with a 10% gap before and after intravenous gadolinium administration. Public databases were used as controls.

Sanger and exome sequencing for variant calling and copy number variant analysis

Genomic DNA samples were obtained from peripheral blood or oral mucosa smears from patients and control individuals by salting out technique (12). To conserve DNA, given that in some cases the patient is no longer available for further collection, we chose to perform DNA amplification using the RepliG kit (Qiagen) following the manufacturer's instructions in all samples from 145 patients.

Intronic primers were designed to evaluate the entire coding region of the *CDH2* gene. (ENSEMBL accession number: ENSG00000170558) (Supplementary Table 1, see section on supplementary materials given at the end of this article). For Sanger sequencing, 16 exons of *CDH2* were well amplified by PCR, sequenced, and analyzed.

The functional impact of the variants detected by Sanger herein was predicted by 13 different types of *in silico* analyses: PolyPhen (http://genetics.bwh.harvard.edu/pph2/index.shtml), Mutation Taster (http://www.mutationtaster.org/), Mutation Assessor (http://mutationassessor.org/r3/), SHIFT, FATHMM, DANN, LIST-S2, DEOGEN2, EIGEN, MVP, REVEL, M-CAP, and PrimateAI. Analysis of the 5' UTR allelic variant was done by the Jaspar program (http://jaspar.genereg.net/).

Isolated proband WES was performed at the University of Michigan DNA Sequencing Core facility (Ann Arbor, MI, USA). Library preparation was performed as described by Roche's protocol for NimbleGen V3 kit and HiSeq 2000 was used for sequencing samples. Alignment to reference genome h19 and variant calling were performed using BWA and GATK, respectively. Annotation was done using ANNOVAR v.2016-02-01. Variants were filtered according to base depth (>10) and genotype quality (≥20). Variant prioritization considered gene regions, specifically those in exonic and splice site regions. Genotype filtering for the isolated proband was applied for an autosomal inheritance model, searching for homozygous variants, or autosomal dominant, for genes already described as a cause for hypopituitarism.

Trio WES was performed at laboratory Fleury (São Paulo, SP, Brazil) with Illumina's NovaSeq 6000 platform. Library preparation was performed as described by Twist Bioscience's protocol. For quality check and alignment to genome reference hg19, Illumina's software Dragen was used. FreeBayes v1.3.2 and ANNOVAR v.2019-10-2 were used for variant calling and annotation, respectively. Variants were first filtered according to the base depth (>10 reads) and genotype quality (≥20). Variant prioritization considered the gene region, preferably ones in exonic or splice site regions. Common variants in 1000G, gnomAD, ABraOM, or SELAdb data sets were excluded, leaving only variants with an MAF of ≤1% (13, 14, 15, 16). Lastly, genotype filtering was applied considering inheritance models for autosomal recessive (homozygous and compound heterozygous), autosomal dominant, *de novo*, and X-linked patterns.

The analysis of copy number variations was performed by Fleury using a control panel of normal samples generated by the laboratory. For this analysis, Illumina's DRAGEN copy number variation pipeline was used, which detects alteration from three contiguous exons (11).

CDH2 variant effects on splicing were evaluated by bioinformatic analysis and cDNA amplification

The web site https://bio.tools/human_splicing_finder was used for bioinformatic analysis. To check for alternative splicing site leading to truncated or frame-shifted protein, cDNA amplification reaction (Supplementary Table 2) and thermal cycling conditions (Supplementary Table 3) from the patient were performed using the sequences of primer F: GTGCATGAAGGACAGCCTCT and primer R: AGCTTCTGAATGCTTTTTGGGA. The PCR fragment is expected to be around 2600 bp.

Cell aggregation assay using stable cell lines expressing human CDH2

L1 mouse fibroblast line (ATCC CRL-2648) was cultured under standard conditions in Dulbecco's modified Eagle medium (Thermo Fisher #11995-065), 10% fetal bovine serum (Corning #35-016-CV), and 1% penicillin-streptomycin (Thermo Fisher #15140122), at 37°C with humidified air supplemented with 5% v/v CO_2. The human *CDH2* open reading frame (NM_001792.4) was cloned and modified by site-directed mutagenesis to create p.V289I (patient variant), p.A77M (negative control), and p.A78M (positive control). Cells were transfected with each of the *CDH2* plasmids and selected with G418.

To assess gene expression in the stable lines, RNA was extracted from confluent p60 dishes of native L1 cells (untransfected) or stable cell L1 lines expressing CDH2 wildtype, A77M, or V289I, reverse transcribed, and assayed for gene expression using Taqman probes for mouse and human CDH2. For analysis of protein expression, 5E4 native L1 cells (untransfected), or stable cell lines expressing CDH2 wildtype, A77M, or V289I were plated onto glass coverslips, fixed, and stained for CDH2 expression with anti-cadherin-2 antibody (polyclonal, raised in rabbit, Abcam #ab12221).

L1 cells were assayed for aggregation based on established protocols (17, 18, 19, 20, 21) except that we excluded EDTA from our protocol (17, 18, 19, 20, 21). This method allowed us to test the homophilic adhesion of L-cells via CDH2 and test the patient variant $CDH2^{V289I}$. Briefly, cells were incubated overnight in lipophilic dyes, DiI, red, Thermo Fisher #D3911) or DiO, green, (Sigma #D4292). The next day cells were combined and incubated in 1 mM calcium chloride with shaking for 2 h, and then fixed and imaged. Five images per sample were quantified with Image J (NIH), and the percent aggregation was calculated as $(N_{0\ min} - N_{120\ min})/N_{0\ min}$. Variances were tested with Shapiro–Wilk and Levene tests, and groups were compared either with one-way ANOVA and Scheffe *post hoc* test or Mann–Whitney *U*-test using SPSS Statistics 25 (IBM).

For more details see supplementary methods.

Luciferase assay for effects on beta catenin

The cells were transfected using 0.75 μL Lipofectamine 3000 (Invitrogen) and 500 ng of plasmids for either wildtype or mutant CDH2 regions on ppGS Cite Neo backbone varying from 2.5 to 80 ng along with a fixed amount of 50 ng beta catenin (pCMV6-XL5 OriGene technologies, Rockville, MD, USA) cloned with CTNNB1 (NM_001904.20) and 100 ng TOPO flash (Millipore Corporation) completed with ppGS Cite Neo backbone. The assay was performed as previously described, where we showed that increased amounts of a likely pathogenic RSPO1 variant caused decreased activation compared to WT (22). The assays were performed in triplicate at least three times.

cdh2 knockout zebrafish model

To study the effects of cdh2 on pituitary development, we have produced a zebrafish model with a cdh2 knockout gene by genome editing using CRISPR Cas9 method. For details see supplementary data and Supplementary Tables 3, 4, 5, 6, 7, 8, and 9.

Statistical analysis

All analyses were performed in the R environment and graphs were made in the GraphPad Prism 8. First, normality and homocedasticity of the data were evaluated by Shapiro and Levene tests, respectively, and since these parameters were achieved, we proceeded with ANOVA analysis (one- or two-way depending on the number of variables for each analysis).

Results

Genetic analysis of the index patient with hypopituitarism

A 3.9-year-old girl with short stature was evaluated, and deficiencies in GH, TSH, ACTH, LH, and FSH were detected (Table 2). She had an ectopic posterior pituitary lobe, non-visualized stalk, and hypoplastic anterior pituitary

Table 2 Combined insulin-TRH-GnRH stimulation test at diagnosis.

Time (min)	**−30**	**0**	**15**	**30**	**45**	**60**	**90**
Glucose (mg/dL)	77	86	57	26	55	28	88
GH (ng/mL)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Cortisol (μg/dL)	9.4	13.1	12.7	11.6	12.2	11.5	11.8
LH (U/L)	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6
FSH (U/L)	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
TSH (μU/mL)	2.67	2.32	3.12	3.76	3.87	4.12	4.02
PRL (ng/mL)	1.1	0.9	2.8	3.1	2.5	2.1	1.4

Min: minutes, mg: milligram, mL: milliliters, ng: nanograms, dL: deciliters, U: units, L: liters, GH: growth, LH: luteinizing hormone, FSH: stimulating, TSHstimulating hormone.

lobe (Fig. 1). We applied WES for analysis of genetic variants in the index patient. Because her parents were first cousins, we first investigated recessive inheritance. Four genes, *PLA2G4A, ANKRD36B, CDC27,* and *CDH2*, (Table 3) fulfilled the criteria of being rare and expressed in mouse and human pituitary, according to the developing mouse pituitary analysis database and GTEx, respectively. No pathogenic loss or gain copy number variants (CNVs) were found in the chromosomal material. Manipulation of CDH2 activity in fetal pituitary cultures can affect GH hormone secretion, so we focused on *CDH2* (23).

The patient was homozygous for *CDH2* (NM_001792.5, [hg19] chr18:25583116, rs142589795) c.865G>A (p.Val289Ile), while her unaffected siblings and parents were heterozygous for this variant Fig. 2. This allelic variant is predicted to have a deleterious effect on function according to several impact prediction algorithms. Allelic variants in *CDH2* have never been associated with hypopituitarism before.

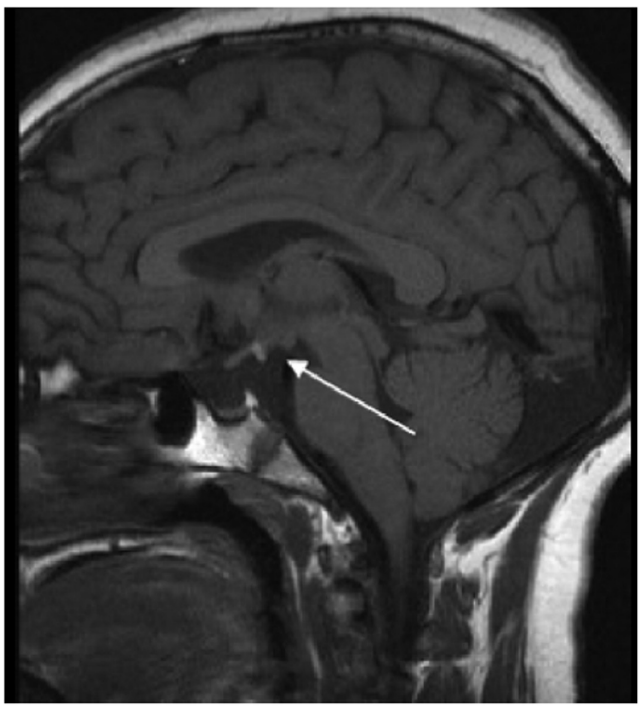

Figure 1
Pituitary magnetic resonance image. Ectopic posterior pituitary lobe (arrow), absent pituitary stalk, and anterior pituitary lobe hypoplasia.

No alternative splicing site for CDH2 by bioinformatic analysis and cDNA amplification was found

The bioinformatics approach revealed no significant impact on splicing signals using HGVS Annotation ENST00000269141.8:c.865G>A. The *CDH2* cDNA amplification revealed just one band with the expected size, in the patient and control (Supplementary Fig. 1).

No pathogenic variants were found in the *CDH2* cohort

To search for other patients with variants in *CDH2,* we screened 145 Brazilian patients with congenital hypopituitarism (Table 1) by Sanger sequencing. Thirteen variants were found: four synonymous, four missense, two deletions, and three 5' UTR variants (Supplementary Table 10). Sixty-four patients carried a single variant, five patients carried two variants, and three carried three variants. The variants c.333C>G (p.Thr111=) and c.352G>A (p.Ala118Thr) are probably in cis because the variants were identified in multiple patients, and both variants were always found together. The frequency of synonymous and 5' UTR variants is high in databases (Supplementary Table 10).

Fibroblasts expressing CDH2^{V289I} have impaired ability to form aggregates and CDH2^{V289I} does not impair β-catenin signaling

We generated mouse L1 fibroblast cell lines stably expressing normal human N-cadherin (*CDH2*) or the

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Table 3 Variants identified by genetic analysis of whole exome sequencing of isolated patient and trio.

Genes	Variants	*In silico* prediction	ACMG
PLA2G4A[a]	p.Asn643Lys p.Asn703Lys	7/13 deleterious	VUS
ANKRD36B[b]	p.Thr1003Met	3/8 deleterious	VUS
	p.Val936Ile	0/8 deleterious	VUS
CDC27[b]	p.Asn539Lys p.Asn238Lys	7/12 deleterious	VUS
	p.Asn575Lys p.Asn642Lys p.Asn636Lys p.Asn635Lys p.Arg629Arg p.Ile137Leu p.Ile179Leu p.Ile240Le	3/13 deleterious	VUS
CDH2[c]	p.Val289Ile	3/5 deleterious	VUS
ZNF677[a]	p.Thr525fs	NA	VUS

[a]Variants found in isolated proband exome filtering; [b]Variants found in trio exome filtering; [c]Variant found in both isolated proband and trio filtering. ACMG, American College of Medical Genetics; NA, not available; VUS, variants of uncertain significance.

variants *CDH2*^{A77M} or *CDH2*^{V289I}. Mouse fibroblast cell lines, L (ATCC CRL-2648) and L929 (ATCC CCL-1) do not express endogenous cadherins, and they are well-established for assaying the effects of cadherin expression on cell aggregation, including type-I, mouse, and chicken cadherins such as E-, N-, or P-cadherin (*Cdh1, Cdh2,* or *Cdh3,* respectively) and other, unconventional cadherins (24). The *Cdh2*^{A78M} variant has decreased ability to aggregate L-cells *in vitro* (17). Therefore, we generated a cell line stably expressing the corresponding human *CDH2*^{A77M} as a negative control. We generated L-lines expressing either *CDH2*^{wt} (positive control) or *CDH2*^{V289I}.

To demonstrate human-specific *CDH2* mRNA and protein expression, we performed quantitative PCR and immunocytochemistry (Fig. 3A and B; Supplementary Fig. 2). The housekeeping gene *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase) is expressed in all cell lines including the non-transfected (native), *CDH2*^{wt}, *CDH2*^{A77M}, and *CDH2*^{V289I}, but not in the water (no template control – NTC). The mean C_T and S.D. were 22.22 ± 0.10; 23.68 ± 0.05; 25.11 ± 0.12; 24.19 ± 0.13 and not detected, respectively. Mouse *Cdh2* was not expressed in the NTC, the native, or any of the human *CDH2* expressing stable lines (C_T: not detected). Human *CDH2* is not detected in the NTC and the native line, but it is detectable in all human *CDH2* stable lines. The mean C_T ± S.D. values are 35.58 ± 0.30; 33.93 ± 0.11; 33.81 ± 0.16, respectively. Immunocytochemistry with an anti-CDH2 antibody on the cultured cells showed comparable protein expression with the *CDH2*^{wt}, *CDH2*^{A77M}, and *CDH2*^{V289I} lines, and no CDH2 protein in the native cells. Thus, we established stable cell lines expressing human *CDH2* mRNA and protein.

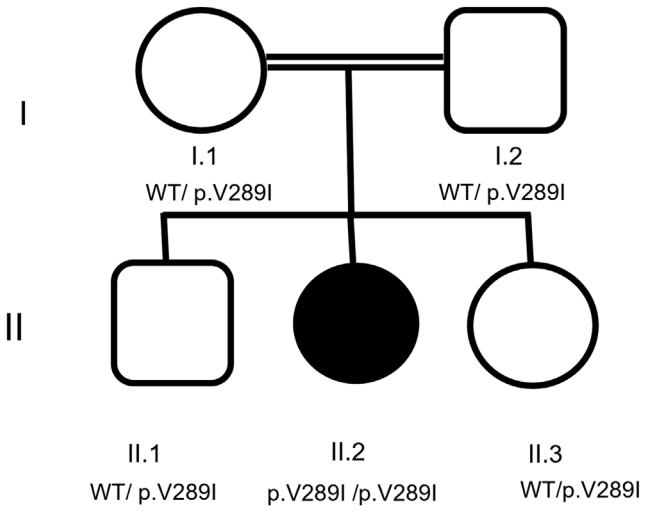

Figure 2
Pedigree of Index patient with *CDH2*^{V289I}. The patient is represented by a black circle. Consanguineous parents and unaffected siblings are heterozygous carriers.

We assessed the capacity of different L-cell lines stably expressing normal *CDH2* or *CDH2* variants to undergo cell aggregation. We stained L-cells with or without *CDH2* variants with either red or green with lipophilic fluorescent membrane dyes. Equal aliquots of red and green cells of the same *CDH2* genotype were mixed and incubated with shaking to permit aggregate formation. Native, *CDH2*^{A77M}, and *CDH2*^{V289I} cells formed very few aggregates, while *CDH2*^{wt} cells formed complex clusters (Fig. 3C). As expected, quantification of the number of cells and aggregates in representative views ($n = 5$) showed that native L1 cells and cells expressing the established A77M mutant formed few aggregates of two or more cells (18.17% ± 6.08 and 10.60% ± 6.25, mean ± S.D.), while cells expressing wt CDH2 efficiently produced aggregates (49.32% ± 6.08) (Fig. 3D). The *CDH2*^{V289I} cells showed poor aggregation (19.08% ± 6.59) that was

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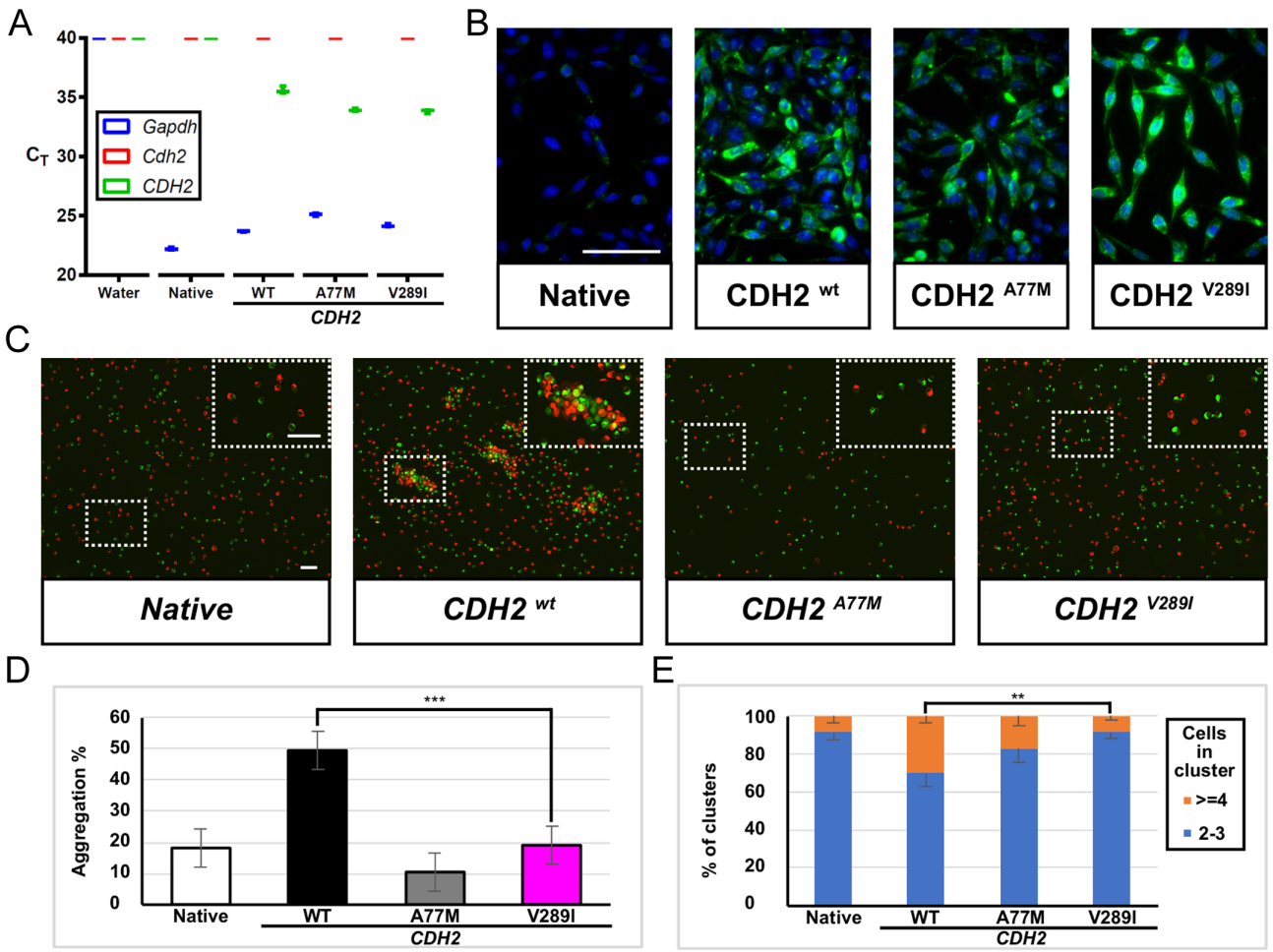

Figure 3

Fibroblasts expressing *CDH2*^{V289I} cannot aggregate *in vitro*. (A) Quantitative PCR with mRNA from mouse L-cell lines stably expressing human *CDH2* variants. Values are marked as mean C_T ± S.D. for each gene and color coded as depicted in the insert. (B) Immunocytochemistry with anti-cadherin antibody (green). Nuclei were counterstained blue with DAPI. All scale bars with white lines in parts (B) and (C) are equal to 100 µm. (C) Aggregation of L-cells with and without *CDH2* variants. Cells were labeled with red (DiI) or green (DiO) fluorescent lipophilic membrane stains the day before the mixing experiment. Equal amounts of red or green labeled native, *CDH2*^{wt}, *CDH2*^{A77M}, and *CDH2*^{V289I} were mixed, aggregated and representative images are shown. Upper right corner inserts are marked with dotted lines in main images and magnified to show individual aggregates. (D) Quantifying the percentage of total cells participating in aggregate formation by CDH2 (mean % ± S.D.; ***P < 0.001). (E) Quantification of number of cells involved in the formation of one aggregate (mean ± S.D.; **P < 0.01). A77M, Ala77Met; V289I, Val289Ile; wt, wildtype.

significantly reduced relative to wildtype *CDH2* (P = 1.4E-5) and indistinguishable from native and *CDH2*^{A77M} cells (P = 0.997 and 0.245, respectively). This demonstrated that *CDH2*^{V289I} cells have decreased ability to form homophilic adhesions via CDH2.

The V289I variant also reduced the number of cells found in each aggregate relative to wildtype CDH2. We quantified aggregates containing only two to three cells or four or more cells (Fig. 3E). The percentages of aggregates with four or more cells per aggregate was greatest for *CDH2*^{wt} (30.38 % ± 3.59), and this was significantly different from *CDH2*^{V289I} (8.93 % ± 2.05, P = 0.008). The percentage of large aggregates in *CDH2*^{V289I} expressing cells was indistinguishable from native and *CDH2*^{A77M} cells (P = 1.0 and 0.548, respectively). Thus, cells with *CDH2*^{V289I} have a much lower propensity to form connections with multiple cells than those with *CDH2*^{wt}.

HEK 293 FT cells transfected with CDH2^{V289I} do not impair β-catenin signaling

To test whether the variant CDH2^{V289I} alters β-catenin signaling, we performed a dual-luciferase reporter assay in HEK 293 FT cells (Invitrogen, Carlsbad, CA, EUA). The CDH2^{V289I} variant did not induce any change in β-catenin signaling (P = 0.87706; Supplementary Fig. 3).

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Zebrafish cdh2 knockout and phenotypic analysis

We produced a zebrafish cdh2 knockout using CRISPR/Cas technology. Sanger sequencing analysis showed that 90% of the genotyped embryos presented genomic alterations in cdh2, such as insertions, deletions, and amino acid changes, indicating the efficiency of the technique. The phenotypic analyses were carried out by observing the animals under a magnifying glass (Nikon), and the animals presented several anomalies such as absence of somites, microphthalmia, malformations throughout the body and head, and cardiac deformity. Mortality analysis was performed by counting live embryos every 24 h. Animals with knockout in the cdh2 gene survived only up to the 10th day post fertilization, with 70% mortality before the 6th day post fertilization (Supplementary Fig. 4 and Supplementary Table 11). These results show that the cdh2 gene is extremely important for the development of the embryo and cannot be completely removed from the body. Therefore, future studies are being developed to generate the animal with the same variant found in the patient, affecting only the EC2 domain of the protein, to characterize the role of this domain in the development of the pituitary gland.

Discussion

The worldwide mutation frequency of *PROP1, POU1F1, HESX1, LHX3,* and *LHX4* mutations in patients with GHD was estimated to be 12.4% and may reach 63% in familial cases from some geographic regions (2). Most patients with CPHD (84.2%) have no genetic diagnosis following screening for the five genes mentioned earlier (3). Recent WES have begun to implicate many additional genes in patients with GHD and related disorders. In the present study, exome sequencing uncovered a homozygous rare variant in *CDH2* in a CPHD patient, p.Val289Ile. *In silico* analysis and functional studies suggest that this variant affects the ability of N-cadherin to promote cell-cell interaction. No other suitable candidate genes were identified in the WES or CNV detection. Additional screening of a large Brazilian cohort with 145 patients did not uncover any additional patients whose condition could be explained by *CDH2* variants. Although we cannot rule out genomic variation or environmental exposures that could contribute to the disorder in the index case, the functional studies make a strong case for considering *CDH2* as a candidate gene in future WES or WGS studies.

Cadherins are transmembrane proteins that have been identified as cell surface molecules responsible for calcium-dependent cell-cell adhesion. *CDH2* encodes cadherin 2, a classical type I cadherin, also known as neuronal or N-cadherin. It has high expression in neuronal tissue in addition to participating in the proliferation and differentiation of neural progenitor cells, in the formation of the neural tube, in neuronal migration and in axon elongation (25, 26, 27, 28, 29,30). Consistent with these pleiotropic functions, abnormalities in critical domains of N-cadherin have been associated with a few human diseases (31, 32).

Based on a study that showed that *CDH2* SNPs conferred an increased risk of canine compulsive disorder, Moya *et al.* searched for *CDH2* heterozygous variants in patients with obsessive compulsive disorder and Tourette disorder. Moya *et al.* found four *CDH2* allelic variants, including the p.Val289Ile. This variant was found in two patients and one normal control. The authors concluded that these heterozygous *CDH2* variants were not disease-causing by themselves and that further studies were necessary (33). This is consistent with our findings as neither heterozygous parent of the proband has these disorders.

More recently, Accogli *et al.* reported that nine subjects harboring *de novo* heterozygous *CDH2* variants had a syndromic neurodevelopmental disorder whose main clinical features are global developmental delay and/or intellectual disability; corpus callosum agenesis or hypoplasia; craniofacial dysmorphisms; and ocular, cardiac, and genital anomalies (34). Most variants were in the EC4-EC5 linker of the EC5 domain of *CDH2* (Fig. 4). It is interesting to note that the frameshift and stop codon variants (p.Leu855Valfs* and p.Leu 856Phefs*) in the C-terminal region in the heterozygous state presented with a more complex phenotype, while the homozygous missense variant in the present study (p.Val289Ile), which is located in the EC2 domain, is associated with hypopituitarism without other malformations. Future studies are necessary to demonstrate why variants in different domains of CDH2 are associated with different phenotypes.

Deletion of the EC2 domain reduces cell migration in fibrosarcoma cells without affecting catenin–cadherin interaction (35). This is consistent with our finding that the missense variant in the EC2 domain does not affect catenin–cadherin interaction. However, variants that reduce cell migration could affect the mesenchymal to epithelial-like transition to pituitary development. Studies are in progress to demonstrate the importance of this protein domain in pituitary development.

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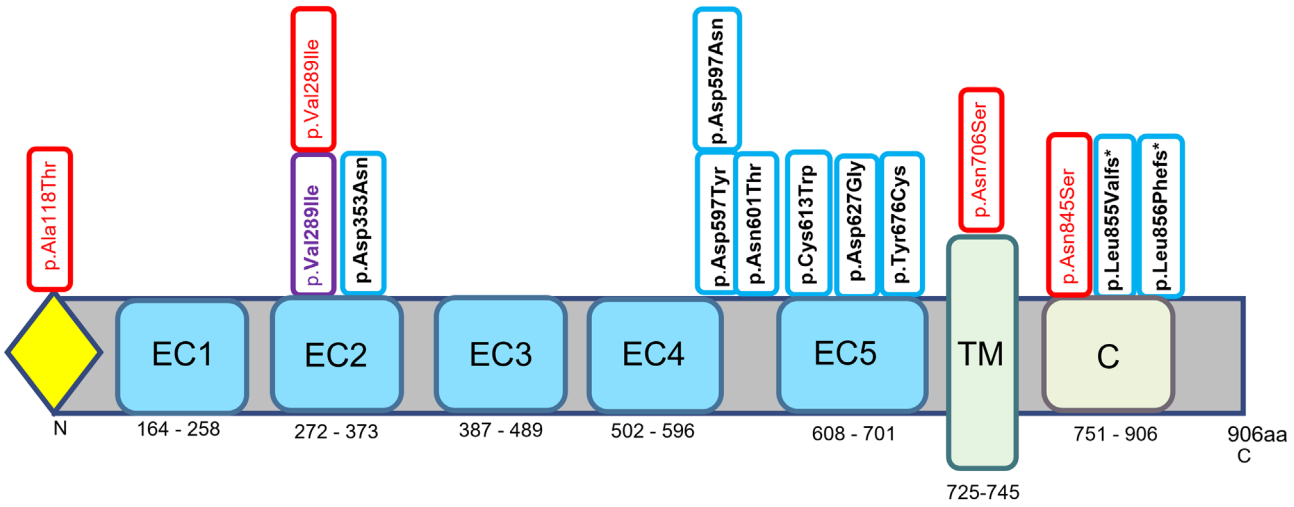

Figure 4
Schematic diagram with allelic variants described in the literature and in the present study with associated phenotypes. The heterozygous allelic variants labeled in red were detected in patients with psychiatric disorders (obsessive-compulsive disorder), but none were thought to be causal. The heterozygous allelic variants labeled in blue are present in individuals with complex phenotype and neurodevelopmental syndromic disorders. The homozygous missense variant, *p. Val289Ile*, labeled in purple, is present in the patient with hypopituitarism. C, cytoplasmic tail; EC1 to EC5, five cadherin extracellular domains; Pro, pro-cadherin region, TM, transmembrane region.

N-cadherin, as stated earlier, is extremely important for the formation of cell aggregates (36), and our *in vitro* experiment shows reduced aggregation of cells expressing p.Val289Ile. Cellular adhesion has an important role during cellular migration (37), and cells expressing N-cadherin with the cytoplasmic domain deleted had reduced traction force (38). Defective cell migration during the epithelium to mesenchymal-like transition might affect pituitary differentiation, cellular organization, and hormone secretion resulting in anterior pituitary hypoplasia and hormonal deficiencies (39, 40, 41). The adenohypophysis originates from a cranial placode, and recent findings showed that during early embryo development, the neural crest cells are attracted to the cranial placode by chemotaxis (chase), while the cranial placode migrates away from these cells by disrupting focal adhesions that generate an asymmetry that promotes its migration (run). The disruption of adhesions is controlled by N-cadherins (42). In addition, *in vivo* experiments with *Xenopus laevis* showed that N-cadherin is also extremely important to create a stiffness gradient that leads to the migration of both neural crest cells and the cranial placode (43). Since our results showed that cells expressing the variant p.Val289Ile have an impaired ability to form aggregates *in vitro*, similar to the well-known p.A78M variant, it is possible that p.Val289Ile variant affects the stiffness gradient and, consequently, impaired migration of cells in the development of the adenohypophyseal placode and neurohypophysis, could contribute to morphological alterations that cause CPHD.

Yuan *et al.* demonstrated by structural comparison that the amino acid change from Val to Ile, a subtle alteration between two hydrophobic residues, which differ from each other by only one methyl group, completely changed the ligand selectivity of a protein. This discovery showcased the beauty and power of structural biology and called for caution in sequence-based functional prediction (44).

Mrozik *et al.* have shown that the stabilization of N-cadherin-mediated adhesion requires the clustering of adjacent monomers on the surface of the same cell, involving the His-Ala-Val (HAV) motif on EC1 and a recognition sequence on the second extracellular domain (EC2) of the lateral N-cadherin monomer (cis adhesion). The membrane expression and lateral clustering of N-cadherin are dependent upon p120 catenin, which localizes N-cadherin at cholesterol-rich microdomains. The initial ligation of N-cadherin extracellular domains triggers the activation of the Rho GTPase family member Rac, which stimulates localized actin filament assembly and the formation of membrane protrusions at points of cell-cell contact (45). The switch from Val to Ile in the present study is only 49 amino acids away from the motif sequence (HAV); if this variant changes the shape of the protein, the HAV motif sequence will not be able to anchor to the other N-cadherin monomer impeding cell-cell adhesion.

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Several studies indicate that β-catenin signaling is regulated by N-cadherins in a wide variety of tissues and functions, such as osteogenic (46) and neuronal differentiation (47, 48), smooth muscle contraction (49), and cancer cell metastasis (50, 51, 52). Canonical WNT signaling with β-catenin is extremely important for pituitary development (53, 54, 55) and for the regulation of gene expression in gonadotropes (56). Our results showed that *CDH2* p.Val289Ile does not affect β-catenin-CDH2 regulation.

CDH2 appears to be a plausible candidate gene for CPHD, and additional unrelated cases are necessary to provide supporting evidence. The ClinGen Gene Curation Working Group recommends that definitive gene-disease association requires an accumulation of convincing genetic and experimental evidence (57). Frequency in population databases, *in silico* analysis, and functional studies supports a link between the p.Val289Ile *CDH2* (N-cadherin) allelic variant and the patient with GH, TSH, ACTH and LH/FSH deficiencies. Further studies are necessary to better understand its role during pituitary development.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/EC-22-0473.

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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Data availability

The data that support the findings of this study are openly available in Sequence Read Archive (SRA) under BioProject ID PRJNA876925, available upon publication.

Author contribution statement

NGBPF: Project design and manuscript writing; NSST: Functional studies using dual luciferase assays, immunohistochemistry analysis, statistics, manuscript writing and review; JLOM: Sanger sequencing protocols and family screening; RK: Sanger sequencing protocols and family screening; JMM: Functional studies using dual luciferase assays and manuscript review; PG: Functional study of CDH2 variant effects on cell adhesion, Manuscript writing; BHVF: Functional study on zebrafish model and manuscript writing; AFFB: Variant calling and filtering from exome sequencing; IPB: Patient's follow up; QF: Filtering variants from exome sequencing, planning CDH2 functional assay; QM: Variant calling and filtering from exome sequencing; ABO: Variant calling from exome sequencing; JZL: Direction of DNA sequence analysis; SAC: Project design, funding, manuscript writing; AALJ: Variant calling and filtering from exome sequencing; BBM: Patient follow up; IJPA: Patient follow up, manuscript writing and revision; LRC: Project design, funding, manuscript writing.

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