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Case Report

Identification of Novel Nucleotide Changes in INHBB Gene by Mutation Screening in Females with Ovarian Dysgenesis: A Case Report

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Abstract

Background: Inhibin and activin regulate the follicle stimulating hormone level by their antagonistic actions and thus have been considered as strong candidate genes in the etiology of ovarian dysgenesis. In the present study, two cases of primary amenorrhea with poorly developed secondary sexual characteristics were reported. The purpose of the study was to identify mutations in candidate gene.

Case Presentation: In this paper, clinical, genetic, biochemical, and molecular findings in female patients with primary amenorrhea were reported. Whole blood culture and G-banding for karyotyping, sequencing, and in silico analysis were performed following the standard protocol. Both cases were cytogenetically characterized as normal females with 46,XX, chromosome constitution. Hormonal assay revealed high level of follicle stimulating hormone and luteinizing hormone. DNA sequence analysis of inhibin identified two novel heterozygous missense mutations of c.975T>A and c.1156G>A which were translated into p.I310N and p.D386N, respectively. These identified positions were highly conserved across species during evolution. In silico prediction tools, intramolecular hydrogen bonding pattern and hydrophobicity analysis, revealed deleterious effect of p.I310N and neutral effect of p.D386N mutation.

Conclusion: Our observation suggested that identified novel mutation in the first case might be the reason for ovarian dysgenesis and provides additional support to the previously reported genotype-phenotype correlations.

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Introduction

Inhibins are dimeric glycoproteins and members of transforming growth factor (TGF)-β family which regulate synthesis and secretion of follicle stimulating hormone (FSH) from pituitary. Inhibin suppresses FSH secretion and lowers concentration of serum inhibin which has been associated with follicular depletion (1) and increased FSH level (Ovarian reserve marker) (2-4). Inhibin exists in two forms of inhibin A and inhibin B, and is composed of heterodimer of inhibin α subunit and two related inhibin β subunits (Inhibin β_A and inhibin β_B, respectively) (Figure 1). Inhibin A suppresses the secretion of FSH from pituitary whereas inhibin B initiates the folliculogenesis (5). Inhibin A is predominantly produced during mid menstrual cycle while inhibin B is produced by granulosa cells during early follicular phase. Interestingly, homodimer of inhibin beta subunit can also form another hormone, activin which exerts opposite effect on FSH synthe-

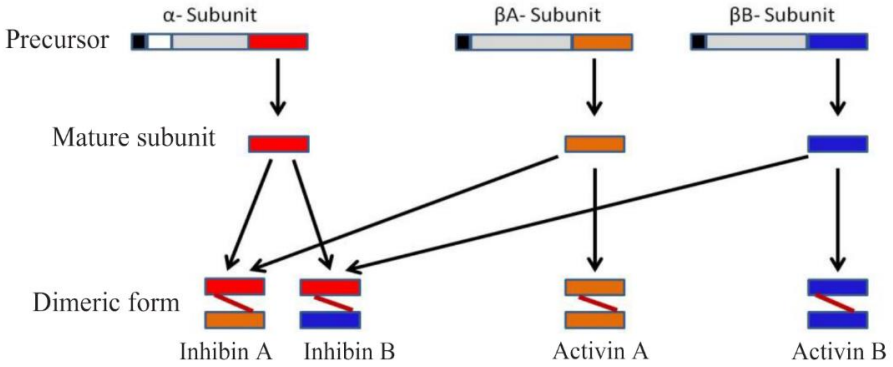

Figure 1. INHA, INHBA, and INHBB produce precursor form of proteins containing N-terminal signal peptide (Black), a propeptide (Gray), and a subunit chain (Red, orange, and blue). These subunits dimerize by disulfide bond to form functional protein

sis and secretion than inhibin and participates in menstrual cycle regulation, cell proliferation, and differentiation (5). Balance of inhibin and activin is required for regulation of reproductive cycle in females (6).

Several studies on infertility reported that over-expression of inhibin α subunit gene in rats results in decreased rate of folliculogenesis with approximately 50% reduction in oocyte maturation that ultimately leads to reduced fertility (7). The study by shelling et al. in 2000 reported all inhibin subunits encoding genes as candidate genes for premature ovarian failure (POF) (8). Furthermore, a missense mutation of p.A257T in INHA serves as a susceptibility factor for POF because it impairs the bioactivity of inhibin B (9). This missense mutation was studied in different ethnic groups by several studies (8, 10, 11).

In the present study, two novel mutations, p.I310N and p.D386N, in inhibin β_B subunit were identified in the cases with primary amenorrhea that might lead to decreased bioactive inhibin level. Decreased inhibin level might result in elevation of FSH by disturbing the negative feedback to pituitary, leading to ovarian dysgenesis.

Case Presentation

Human subjects: Two cases suspected with primary amenorrhea were recruited for the study and they were referred from the Department of Obstetrics and Gynecology, Sir Sunderlal Hospital, Varanasi, India to our centre (Centre for genetic Disorders, Banaras Hindu University, Varanasi, India) in 2016-2017. Clinical history and photographs were collected from each case after receiving informed written consent. This study was approved by institutional ethics committee (No.: F.Sc./Ethics Committee/2015-16/7). Both cases had the following clinical history; the first case was a 17 year old girl with primary amenorrhea along with poorly developed secondary sexual characteristics, breast Tanner stage I, absent pubic and axillary hair. She was diagnosed with absence of uterus during ultrasound scan. The second case was a 21 year old female who had primary amenorrhea along with poorly developed secondary sexual characteristics. Ultrasound showed absence of uterus and ovaries.

Cytogenetic analysis: Whole blood cultures were set in RPMI-1640 medium at pH=7.2 (Sigma-Aldrich, USA) with 10% fetal bovine serum (Himedia, India) and induced by phytohaemagglutinin-M (Sigma-Aldrich, USA). Next, 30-50 metaphases were analyzed by Saline Trypsin Giemsa (STG) method using Ikaros karyotyping system—Metasystems software (Carl Zeiss Microscopy GmbH, Germany). Karyotyping was performed according to ISCN nomenclature.

Hormonal assay: Serum was isolated from each sample which was stored at -80°*C* until use for hormonal assay. Follicle stimulating hormone (FSH) and luteinizing hormone (LH) were measured by chemiluminescence method (Immulite-1000 Analyser, Siemens Healthcare Diagnostics Products, USA) as per the manufacturer's instructions.

Polymerase chain reaction (PCR) and sequencing: All exons and exon-intron boundaries of all subunits of inhibin gene (INHA, INHBA, and INHBB) were amplified and directly sequenced. Primers were designed by primer blast software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). PCR products were sequenced in an automated

Table 1. Primer list used for sequence analysis of INHA, INHBA, and INHBB gene

Primers	Sequences (5'→3')	Product size (*bp*)	Annealing temperature (℃)
INHA_EX1F	TGCGTCAGAGATAGGAGGTCT	531	60
INHA_EX1R	CCATGCTGTGCCTTGCTTTT		
INHA_EX2.1F	CTCCTGCTGAAGAGGAGGG	546	58
INHA_EX2.1R	CGCAGAGCAGAGGGAGAC		
INHA_EX2.2F	CCTGGTGGCCCACACTC	539	62
INHA_EX2.2R	AACTCTGCCTTTCCTCCCAG		
INHBB_EX1F	CAGTGACCCGCGACCTC	797	62
INHBB_EX1R	GAGCTAGCAAGGAGAGGGAG		
INHBB_EX2.1F	AGCAGAGAGTGTGTTTCCCC	513	60
INHBB_EX2.1R	ACTCCAGGCCTCGCTTG		
INHBB_EX2.2F	GTGGACCCAGGCGAAGAG	495	60
INHBB_EX2.2R	GCTGGAAGAAGCCCACC		
INHBA_EX1F	AGTTCCTCCTGGGACTGTCA	700	60
INHBA_EX1R	ACAGCCACAAACCTACAGCA		
INHBA_EX2.1F	AACTCTTGCTCCCTTTCCCC	322	58
INHBA_EX2.1R	ACATCGGCTGGAATGACTGG		
INHBA_EX2.2F	TGACCTTGCCATCACACTCC	663	58
INHBA_EX2.2R	ACAGAAGACTCCTGCTTGCC		

3130 Genetic Analyzer (Applied Biosystems, USA) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's protocol. The identified mutations were screened in 150 control individuals from the same population by Sanger sequencing.

In silico analysis: Obtained nucleotide substitutions were further analyzed using MutationTaster online tool (www.mutationtaster.org) and other prediction tools like predictSNP, SIFT, Panther, SNAP, Polyphen-1, Polyphen-2, PhD-SNP, and MAPP. Hydrophobicity profile and helix-forming properties of wild-type and mutant INHBB protein were also examined by ProtScale program available at the ExPASy web server.

Homology modeling of inhibin β_B subunit: The inhibin β_B subunit was homology modeled by using coordinates from Protein Data Bank. Three protein structures named 3rjr_A, 5hly_A, and 5hlz_A were utilized for modeling using Modeller 9.15 and selection of the best model was done by using four different web servers and tools, *i.e.* RAMPAGE (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) for Ramachandran plot analysis, ERRAT program (http://services.mbi.ucla.edu/ ERRAT/) for verifying protein structures determined by crystallography, PDBsum (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html) for structural analyses, and Modeller 9.15 to find out the best model among all. The figures were produced using standalone tool, DeepView–Swiss-PdbViewer (http://spdbv.vital-it.ch/).

Results

Cytogenetic analysis revealed 46,XX karyotype signifying the normal chromosome constitution in both cases (Figures 2A, 3A). Hormonal assay from serum showed FSH and LH levels of 90.3 and 18.5 *mIU/ml*, respectively in case 1. In case 2, the measured level of FSH and LH was 55.95 and 16.09 *mIU/ml*, respectively. Higher level of both FSH and LH, biomarker of gonadal dysgenesis, was observed in both cases. Resequencing of INHA, INHBA and INHBB genes identified two novel non-synonymous substitutions of c.975T>A in case 1 and c.1156G>A in case 2. These variations were translated into p.I310N and p.D386N, respectively (Figures 2B, 3B). Both isoleucine at position 310 and aspartic acid at 386 position were also highly conserved across species during evolution except a few of them (Figures 2C, 3C). P.I310N variation was predicted to be pathogenic by several in silico prediction tools (Mutation Taster, SIFT, SNAP, Polyphen-1, Polyphen-2, PhD-SNP, MAPP and predictSNP) (Figure 2D) while p.D386N mutation was predicted as neutral (Figure 3D).

The study of intramolecular hydrogen bonding pattern of p.I310N mutation revealed the formation of three new hydrogen bonds in mutant pro-

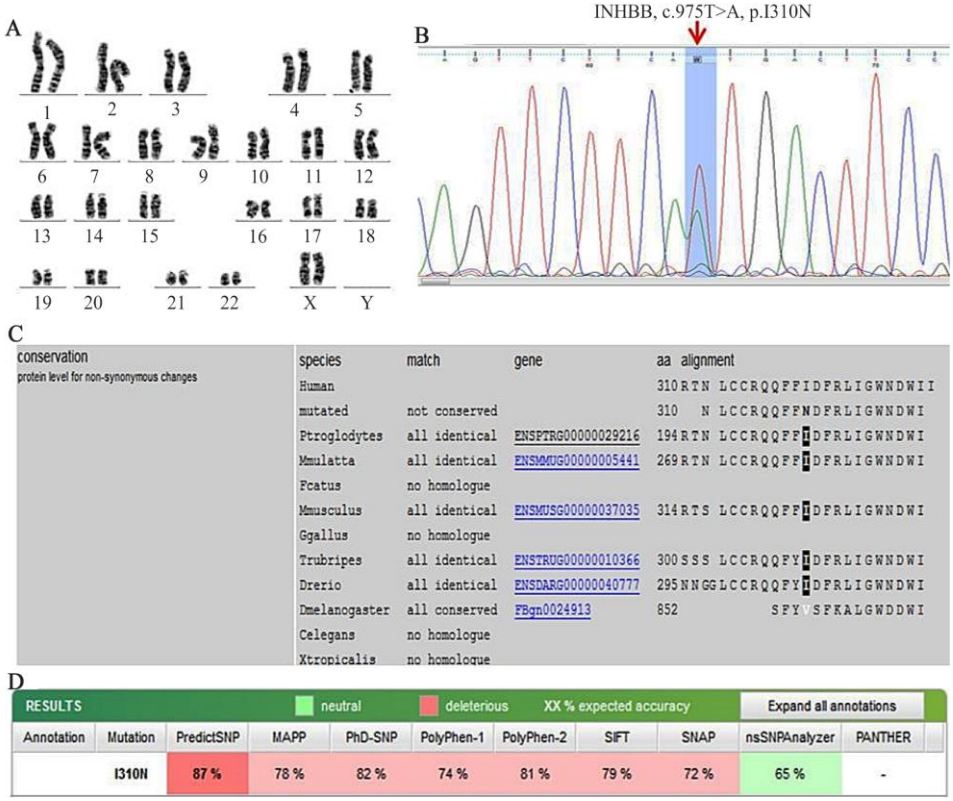

Figure 2. A) Representative karyotype (46,XX) of case 1, B) Electropherogram of the c.975T>A (p.I310N), C) The multiple sequence alignment of the p.I310N mutant protein reveals the conservation of isoleucine at this position throughout evolution, D) In silico prediction of both variants using prediction tools of SIFT, SNAP, Polyphen-1, Polyphen-2, PhD-SNP, MAPP, and PredictSNP

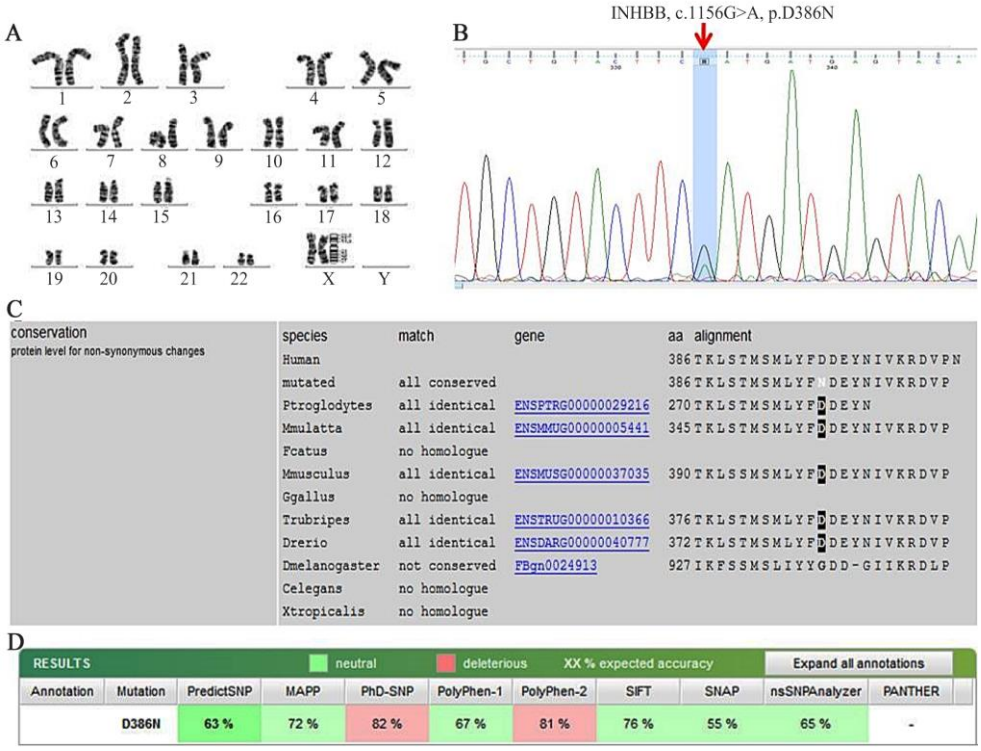

Figure 3. A) Representative karyotype (46,XX) of the second case, B) Electropherogram of the c.1156G>A (p.D386N), C) The multiple sequence alignment of the p.D386N mutant protein reveals the conservation of isoleucine at this position throughout evolution, D) In silico prediction of both variants using prediction tools of SIFT, SNAP, Polyphen-1, Polyphen-2, PhD-SNP, MAPP, and PredictSNP

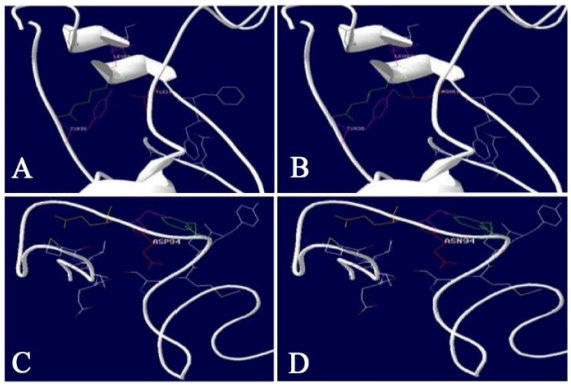

Figure 4. The tertiary structure of the INHBB protein predicted by Modeller and visualized on the standalone tool DeepView–Swiss-PdbViewer, A) the wild-type INHBB protein with I310, B) the mutant N310 INHBB protein formation of three new hydrogen bonds occurs with TYR327, ARG313, and LEU314, C) the wild-type INHBB protein with D386, B) the mutant N386 INHBB protein shows no change in hydrogen bonding pattern

tein with TYR327, ARG313, and LEU314 (Figures 4A, 4B). The analysis of alteration in hydrophobicity of the protein due to this mutation revealed minor changes in protein microenvironment (Figures 5A, 5B). On the other hand, intramolecular hydrogen bonding pattern and hydrophobicity analysis of p.D386N mutation showed the neutral effect (Figures 4C, 4D, 5C, 5D).

Discussion

The present study reports two mutations (p.I310N and p.D386N) in inhibin β_B subunit, identified in females with primary amenorrhea. The first identified mutation results in alteration in intramolecular hydrogen bonding pattern as well as protein microenvironment due to hydrophobicity change while the second mutation does not show any pathogenic effect. The alteration in protein microenvironment might interfere with normal function of inhibin B protein that leads to disturbance in inhibin and activin balance. Activin and inhibin balance plays an important role in regulation of ovarian cycle through FSH level. As inhibin β_B subunit involves in multiple complexes with antagonistic functions, the consequences of mutations related to loss-of-function or gain-of-function of protein might be difficult to predict. However, there is no report till date that mutation

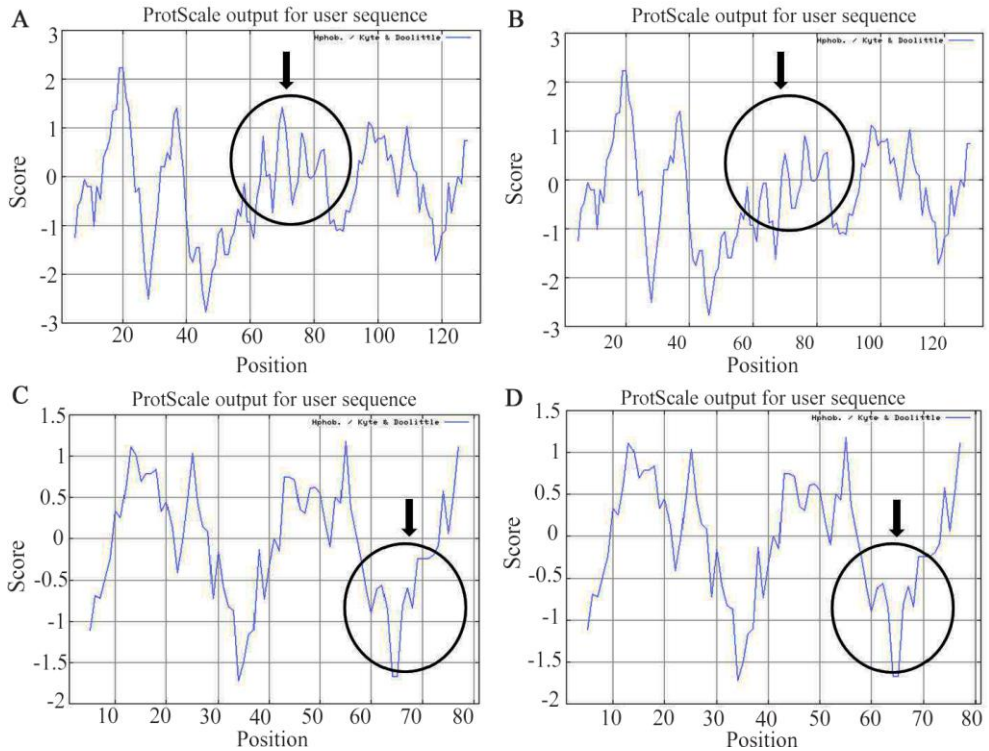

Figure 5. Hydrophobicity profile of wild-type and mutant p.I310N and p.D386N in inhibin β_B subunit predicted by the ProtScale program at the ExPASy server, A) the black circle represents the hydrophobicity around I310 in the wild-type protein, B) the black circle represents the hydrophobicity around N310 in the mutant protein showing a mild shift in hydrophobicity compared to the wild-type, C) black circle represents the hydrophobicity around D386 in the wild-type protein, B) the black circle represents the hydrophobicity around N386 in the mutant protein showing no shift in hydrophobicity compared to the wild-type

in inhibin β_B subunit is involved in ovarian dysgenesis in females. An independent study reported that p.M370T substitution is involved in spermatogenic impairment in males (12). Furthermore, very low level of inhibin B in prepubertal girls is a biomarker for premature ovarian failure (13, 14). INHA has been more studied in ovarian failure and several mutations have been reported by different studies (10, 11, 15-17).

A nucleotide substitution (INHA 769G>A) in inhibin α subunit is well studied in different populations with reference to premature ovarian failure (8, 11, 15-17). Dixit et al. also reported three different mutations (p.S92N, p.H175Q, p.A182D) in INHA gene in patients with premature ovarian failure (10). A p.H175Q mutation in INHA was observed by Dixit et al. in a patient who experienced menopause at the age of 30 years with high level of FSH and LH (78 *IU/l* and 47 *IU/l*, respectively). Interestingly, another patient of this study with p.A182D mutation in INHA never menstruated and had underdeveloped secondary sexual characteristics.

Conclusion

Our study has demonstrated for the first time that mutation in INHBB subunit might lead to ovarian dysgenesis. Mutations in inhibin gene might affect the FSH level either through inhibition by inhibin or stimulation by activin through disturbing the ratio between inhibin and activin. Inhibin is suggested as a marker for ovarian reserve in premature ovarian failure patients and early screening of inhibin gene is necessary for identification of ovarian reserve to manage ovarian failure.

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Conflict of Interest

The authors have no conflict of interest.

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