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RESEARCH ARTICLE

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Multilevel Annotation of Germline *MEN1* Variants of Synonymous, Nonsynonymous, and Uncertain Significance in Indian Patients With Sporadic Primary Hyperparathyroidism

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ABSTRACT

Primary hyperparathyroidism (PHPT) is third most common endocrine disorder characterized by hypercalcemia with elevated or non-suppressed parathyroid hormone levels by parathyroid tumors. Familial PHPT, as part of multiple endocrine type-1, occurs due to the germline mutation in the *MEN1* gene. The involvement and the role of germline *MEN1* variations in sporadic PHPT of Indian PHPT patients are unknown. Precise classifications of different types of MEN1 variations are fundamental for determining clinical relevance and diagnostic role. This prospective cohort study was performed on 82 patients with PHPT (with no clinical or history of *MEN1*) who underwent screening for *MEN1* variations through Sanger sequencing. Multilevel computational analysis was performed to determine the structure–function relationship of synonymous, nonsynonymous, and variants of uncertain significance (VUS). Of the 82 PHPT patients, 42 (51%) had 26 germline *MEN1* variants, including eight nonsynonymous, seven synonymous, nine VUS, one splice site, and one regulatory variation. Five most common germline variations (c.1838A>G, c.1817C>T, c.1525C>A, c.-35A>T, and c.250T>C) were observed in this study. c.-35A>T (5′ untranslated region [UTR]) was associated with recurrence of PHPT (odds ratio [OR] = 5.4; $p = 0.04$) and subsequent detection of other endocrine tumors (OR = 13.6, $p = 0.035$). c.1525C>A was associated with multi glandular parathyroid tumor (OR = 13.6, $p = 0.035$). Align–Grantham variation and Grantham deviation (Align-GVGD), functional analysis through hidden Markov MODEL (FATHMM), and MutationTaster analysis reported the disease-specific potential of VUS and synonymous variations. Significant linkage disequilibrium was observed in c.1785G>A and c.1817C>T ($r^2 = 0.3859$, $p = 0.0001$), c.1475C>G and c.1525C>A ($r^2 = 0.385$, $p = 0.0004$), and c.1569T>C and c.1838A>G ($r^2 = 0.488$, $p = 0.0001$). The detection of *MEN1* variations, especially those with disease-specific potential, can prompt early screening for other MEN1-related tumors and disease recurrence. © 2022 American Society for Bone and Mineral Research (ASBMR).

KEY WORDS: HYPERCALCEMIA; MULTIPLE ENDOCRINE NEOPLASIA TYPE 1 (MEN 1); PARATHYROIDECTOMY; PARATHYROID CARCINOMA; NEOPLASM; PARATHYROID GLAND; PRIMARY HYPERPARATHYROIDISM (PHPT); VARIANTS OF UNCERTAIN SIGNIFICANCE (VUS)

Introduction

Primary hyperparathyroidism (PHPT) is characterized by hypercalcemia with elevated or nonsuppressed parathyroid hormone (PTH), caused by parathyroid adenoma, hyperplasia, and rarely carcinoma.[1] The reported prevalence of PHPT varies from 3 per 1000 in the general population to as high as 21 per 1000 in postmenopausal women.[2] Our understanding of the

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genetic basis of the disease is currently restricted to the familial forms of PHPT, like Multiple Endocrine Neoplasia 1 (MEN1) and 2Asyndrome (MEN2A), isolated familial PHPT, and hyperparathyroid-jaw tumor(HPT-JT) syndrome.[3] However, the most common form of familial PHPT is seen in patients with MEN1.[3] The genetic etiology of familial *MEN1* syndrome has been confined to the *MEN1* gene (OMIM 613733),[4] located on the long arm of chromosome 11 (11q13).[5] The gene product of *MEN1*, menin (610–amino acid nuclear protein), possesses dichotomous functions by regulating both positive and negative gene expression.[6] In addition, menin interacts with multiple transcription factors like JunD, nuclear factor kappa B (NF-κB), SMAD family member 3 (Smad3), Replication protein A 32-kDa (RPA2), Fanconi anemia group D2 (FANCD2), histone deacetylase 1 (HDAC1), Apoptosis signal-regulating kinase 1 (ASK), and checkpoint suppressor 1 (CHES1),[6,7] which are essential for physiological cellular processes.

Most PHPT patients are older, relatively asymptomatic, and do not have a positive family history and thus are considered sporadic cases.[8,9] However, the disease tends to occur at a much younger age and tends to be more severe in the Indian subcontinent, as has been reported by our group.[10]

MEN1, the most commonly mutated gene, has been widely studied in western PHPT patients. However, the prevalence of genomic *MEN1* mutations in nonfamilial PHPT from India is unknown. Because Indian PHPT patients present at a young age, the prevalence of *MEN1* mutations might be expected to be high in Indians. Early identification of pathogenic *MEN1* mutations can enable timely screening for other MEN1-related tumors, notably pituitary adenomas, pancreatic neuroendocrine tumors, and carcinoids. Notably, pancreatic neuroendocrine tumors and thymic carcinoids have malignant potential in MEN1; hence, early identification can prompt timely institution of appropriate surveillance and therapy. In addition, identifying *MEN1* mutation in a proband can prompt genetic screening in other clinically unaffected family members. Unfortunately, there is a lack of information regarding the MEN1 variations in sporadic nonfamilial Indian PHPT.

Multiple *MEN1* variations have been reported to date, but the proper characterization of variations in terms of disease potential is lacking.[8] Furthermore, information regarding the functional consequences of synonymous, pathogenic, variants of uncertain significance (VUS), and regulatory variations is still lacking in PHPT. However, characterizing the functional consequences of variations by laboratory experimentation is time consuming and expensive. Therefore, multiple computational prediction algorithms can predict putatively functional variants for further investigation. Consequently, we believe there is a need for disease-specific variant predictions. Considering the paucity of aforementioned data in the existing literature, we sought to identify and characterize *MEN1* genetic variants in clinically apparent nonfamilial Indian PHPT patients. We also investigated the effects of variants on messenger RNA (mRNA) and protein structure and their associations with clinical manifestations of PHPT, the risk for multiglandular disease (that might dictate surgical approach), and subsequent detection of other *MEN1*-related tumors.

Patients and Methods

Participant recruitment

We conducted a prospective cohort study wherein consecutive PHPT patients attending the Department of Endocrinology at the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, were screened for possible inclusion in the study between June 2014 and January 2019. The diagnosis of PHPT was based on the documentation of hypercalcemia (albumin adjusted serum calcium > 10.2 mg/dL, with plasma intact parathyroid hormone (iPTH) level of more than 20 pg/mL along with the demonstration of a parathyroid mass on neck ultrasonography and technetium sestamibi (^{99m}Tc-SestaMIBI) scan and histopathological confirmation of a parathyroid adenoma/hyperplasia/carcinoma at the surgery. In addition, recurrence of PHPT was defined as the recurrence of PTH-dependent hypercalcemia after at least 6 months of biochemical remission following parathyroidectomy.[11]

A thorough family history (including first-, second-, and third-degree relatives) was elicited in all the patients during recruitment. Specific family history points included the history of renal stone disease, parathyroid/neck tumors, neck surgeries, elevated calcium levels, pituitary/brain tumors, brain surgeries, abdominal/pancreatic tumors, and abdominal surgeries. Two investigators (SKB and RP) obtained family history independently. In addition, a thorough personal history of other *MEN1*-related tumors was elicited. Finally, PHPT patients without a significant family or personal history of MEN1 were recruited in the present study. Patients with parathyroid carcinoma were excluded. The follow-up data of recruited subjects were obtained from the outpatient department.

We also selected age- and sex-matched healthy controls for the study. Controls were selected from family members of patients (unrelated to those cases included in the present study) attending the outpatient services of the Department of Endocrinology at our institute. A thorough medical history (including the history of renal stone disease, pathological fractures, acute pancreatitis, elevated serum calcium, brain tumors, and abdominal tumors) was elicited to rule out any underlying medical comorbidities. Apparently, healthy subjects without any significant medical history underwent blood investigations, including serum calcium, phosphate, albumin, alkaline phosphatase (ALP), creatinine, iPTH, and vitamin D. Finally, those with normal biochemical profiles were included in the study as healthy controls. Institutional Ethics Committee (INT/IEC/2017/334) of PGIMER, Chandigarh, India, approved the study protocol. Written informed consent was obtained from all the participants.

The overall workflow of this study population is given in Fig. 1.

Biochemical parameters

As per institutional protocol, blood samples for biochemical investigations were collected after 8 hours of the overnight fast. Baseline investigations at the initial diagnosis were performed before vitamin D supplementation. Serum calcium (reference range [RR] 8.6–10.2 mg/dL), albumin (RR 3.4–4.8 g/dL), inorganic phosphate (RR 2.5–4.5 mg/dL), ALP (RR 40–129 IU/L), creatinine (RR 0.5–1.2 mg/dL), and fasting blood glucose were measured by autoanalyzer (Olympus, Waltham, MA, USA; COBAS-8000). Calcium values were corrected for the respective serum albumin levels. Plasma iPTH (RR 15–65 pg/mL) and 25-hydroxyvitamin D (RR 11.1–42.9 ng/mL) were measured by electrochemiluminescence assay using commercially available kits (Elecsys 2010 system; Roche Diagnostics, Mannheim, Germany).

Identification of *MEN1* variations

Genomic DNA was isolated from peripheral blood leukocytes of the 82 PHPT patients and 12 controls using a Qiagen DNA

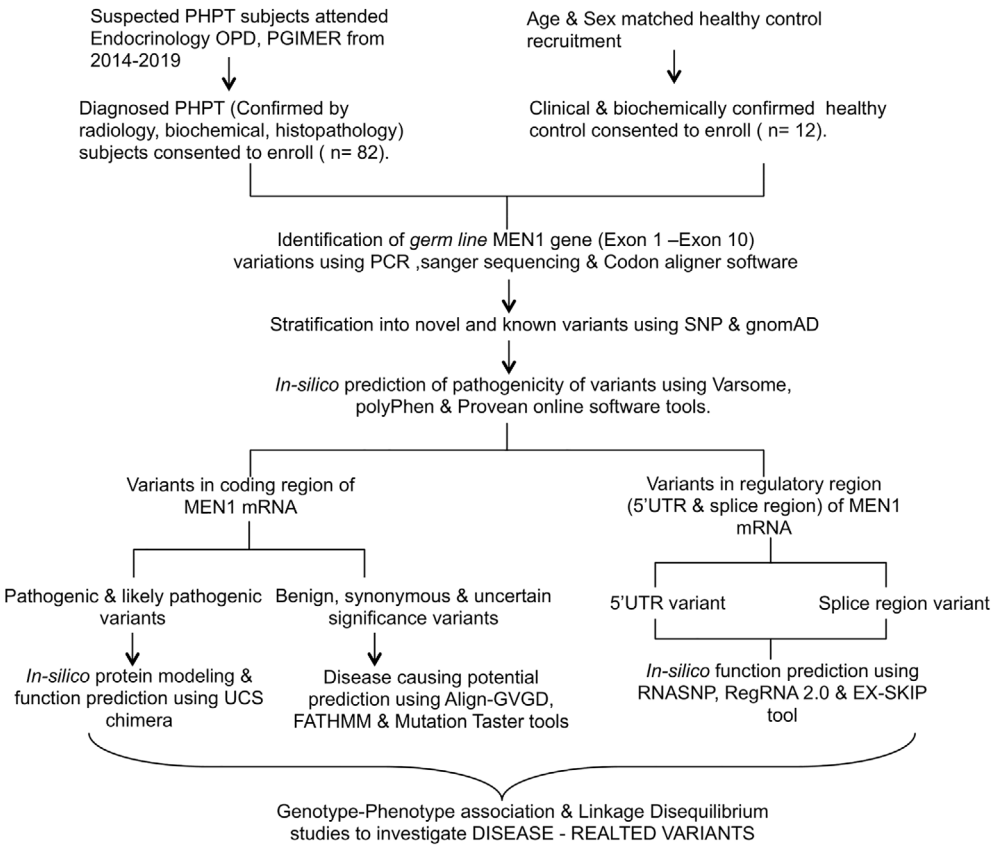

Fig. 1. An overview of the experimental design depicting recruitment criteria of study subjects, MEN1 mutations screening, in silico characterization of variants and validation of variants and genotype–phenotype association and linkage disequilibrium studies of variants.

isolation kit (QIAGEN, Valencia, CA, USA; Cat#51104) and subjected to polymerase chain reaction (PCR) amplification of all the coding exons of the *MEN1* gene using 100–200 ng genomic DNA, 1.25mM $MgCl_2$ (Solis Biodyne, Tartu, Estonia), 0.25mM deoxynucleoside triphosphates (dNTPs) (Solis Biodyne), 20pM of each primer, and 0.5 units of Taq polymerase (Solis Biodyne; Cat#01-01-00500) in a 25-µL volume mixture using a Bio-Rad thermocycler (Bio-Rad Laboratories, Hercules, CA, USA). The primer pair for each exon was given in Table S1. For most of the exons, the PCR program started with denaturation at 95°C for 5 minutes, followed by 35 cycles which contained cycle denaturation at 94°C for 1 minute, annealing at 60°C for 45 seconds, extension at 72°C for 1 minute, and ended with a final extension at 72°C for 7 minutes. The respective standardized annealing temperatures of each exon are given in Table S1. The amplified PCR products encompassing different flanking regions and exons of the *MEN1* gene were purified using Qiagen kits (QIAGEN: Cat#28106) and were sequenced using ABI genetic analyzer (Applied Biosystems [aka ABI], Foster City, CA, USA; Model: ABI-3730XL). The obtained nucleotide sequences were compared with genomic sequences of the *MEN1* gene (id NG_008929.1) from NCBI Gene (https://www.ncbi.nlm.nih.gov/gene). All the chromatograms were visualized and analyzed by the CodonCode Aligner (https://www.codoncode.com/aligner/). Mutations were confirmed against known natural variants of the *MEN1* gene. The database/source for searching known MEN1 variants was dbSNP NCBI (https://www.ncbi.nlm.nih.gov/snp/) and VarSome (https://varsome.com).

Linkage disequilibrium analysis

We selected nine variants (variants present in more than one subject or co-occurring with other variants in the same subjects) of *MEN1* in nonfamilial PHPT. So, based on the genotype data of the genetic variations, linkage disequilibrium (LD) analysis was performed using HaploView software version 4.2 (Broad Institute, Cambridge, MA, USA). LD addresses the co-inheritance of different single nucleotide polymorphisms (SNPs).[12]

In silico analysis of variants

Interpretation of mutation

The standards and guidelines of the American College of Medical Genetics and Genomics (ACMG) were followed to interpret the sequence variants.[13] Each variation was analyzed and compared with already available information on *MEN1* variants. Two databases verified novel variants: The Genome Aggregation Database or gnomAD (https://gnomad.broadinstitute.org) and VarSome. The variations were also categorized based on their functional effects on the menin. The VarSome database predicts the pathogenic impact of mutation based on the cumulative pathogenicity score of more than 15 databases like

MutPred (http://mutpred.mutdb.org/), FATHMM (http://fathmm.biocompute.org.uk/), DANN (https://cbcl.ics.uci.edu/public_data/DANN/), etc.[14]

Prediction of MEN1 *variants pathogenicity*

The potential pathogenic effect of all known and novel variants was analyzed using multiple bioinformatics tools to increase the prediction confidence level. In this study, we consulted Protein Variation Effect Analyzer (PROVEAN) (http://provean.jcvi.org), Polymorphism phenotyping V2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/pph2/), and VarSome. These bioinformatics tools could predict whether an amino acid substitution or deletion may affect protein function based on sequence homology and physical properties of substituted amino acids. The computational in silico analysis using the PROVEAN score could predict the deleterious nature of nonsynonymous or indel variants based on the threshold score of −1.00 to −4.10 with 90% sensitivity. The identified variants were subjected to PROVEAN software to obtain scores that would predict whether an amino acid substitution or insertion/deletion impacts the biological function of the menin protein.[15] PolyPhen-2 would classify variants into three categories: probably damaging, possibly damaging, and benign (neutral). The PolyPhen-2 score range between 0.5 and 0.99 was considered damaging.[16] Based on the prediction by the databases mentioned above, the effect of each mutation in this study was categorized as benign, likely benign, pathogenic, likely pathogenic, and VUS (Table 1). The effect of a mutation in the splice site region was predicted by the web-based EX-SKIP tool (https://ex-skip.img.cas.cz/), which compares the exon splicing enhancer/exon splicing site profile of a wild-type and mutated allele to predict the chance of exon skipping.[17]

Prediction of protein structural alterations in pathogenic nonsynonymous variants

To predict the effect of pathogenic nonsynonymous variants on the structural stability of menin, we used the UCSF Chimera program (https://www.cgl.ucsf.edu/chimera/). This program would predict the atomic contacts (ie, steric clashes) of mutated amino acids with their side chains. The mutated amino acids with different rotamers lead to different steric clashes that would affect the stability of the menin protein.

Biophysical validation of VUS

To fully understand the role of VUS MEN1 variants, the Align–Grantham Variation and Grantham Deviation (Align-GVGD) scores (http://agvgd.hci.utah.edu/) online tool was used. Align-GVGD combines the biophysical characteristics features of amino acids such as side chain composition, the polarity of amino acids, and protein multiple sequence alignments (GV and GD scores) to anticipate the effect of VUS. The sequence of menin protein (NP_000235) and VUS were given as input information for each program and default parameters of assigned programs were executed. The analysis is based on GV and GD scores (0 to >200) and graded classifiers (C0 to C65).

Functional analysis of VUS

FATHMM is a high-throughput web-server that predicts the functional consequences of *MEN1* VUS. The details of menin protein (NP_000235) and VUS were given as input under the disease-specific coding variants panel, and the program was executed. The FATHMM analysis designates a prediction score under the disease-specific algorithm. A prediction score of less than zero indicates a chance the mutation is associated with a disease of interest, with lower scores indicating increased confidence in the association.

Disease-causing potential of VUS and synonymous variants

The effect of synonymous and VUS was analyzed using the MutationTaster tool (http://www.mutationtaster.org/).[18] This web-based server identifies disease-causing potential based on evolutionary conservation, changes in regulatory features, mRNA splicing, and posttranslational modifications. The *MEN1* transcript ID (ENST00000443283), base change position, and mutant base were given as input information. The results were automatically analyzed using the Bayes classifier to predict the disease potential.

Prediction and analysis of RNA structure aberration and transcriptional regulatory motif

The effect of variants in UTRs on RNA secondary structures was determined by the RNAsnp tool (http://rth.dk/resources/rnasnp). In brief, RNAsnp computed the global RNA folding pattern that would predict the effect of SNPs on short RNA sequences (<1000 nucleotides [nt]), where the base pair probabilities of the wild-type and mutant RNA sequences were also calculated. In addition, the structural difference between wild-type and mutant were calculated using Euclidean distance or Pearson correlation with the corresponding *p* value. Finally, for the analysis of the transcriptional motif, the wild-type, and mutant-type sequences were retrieved and screened using the bioinformatics tool RegRNA 2.0 (http://regrna.mbc.nctu.edu.tw/).

Genotype–phenotype and haplotype analysis

The genotypes and allele frequencies for variations were stratified for wild-type, heterozygosity, and homozygosity of the allelic variant. The OR measured association within two categorical variables with 95% confidence intervals (CIs). The haplotype analysis was done by Haploview software (https://www.broadinstitute.org/haploview/haploview). Statistical analysis was performed using SPSS software (version 13.0; SPSS Inc., Chicago, IL, USA). Discrete and continuous variables were compared between patients and controls using an unpaired *t* test and chi-square test/Fisher's exact test.

Screening for other *MEN1*-related tumors in patients with germline *MEN1* mutations

In all the patients in whom we had identified *germline* MEN1 mutations, we have also performed biochemical and radiological screening for other MEN1-related tumors. Notably, we performed serum prolactin, insulin growth factor 1 (IGF1), chromogranin A, contrast-enhanced magnetic resonance imaging (MRI) sella, and contrast enhanced computed tomography (CECT) chest and abdomen.

Results

A total of 134 patients with PHPT visiting the Department of Endocrinology at PGIMER were screened within the prespecified period. Of the134 patients, 82 were included in the study (35 did not provide consent, seven had a significant family history, four had parathyroid carcinomas, four had another MEN1-related tumor at presentation, and two were genetically proven MEN1

Table 1. Frequency of Germline Variants, Protein Change, and Pathogenicity Identified in *MEN1* Gene (PHPT Subjects = 42)

Splice region	Germline variant/ variant	Protein change	Novel	Loci number	Pathogenicity	Genotypic frequency**	Allelic frequency**	gnomAD allele frequency (overall) (Asian)	
	Germline variant								
1	c.-35A>T[$]	5′UTR	rs679946	64578113	Benign	AA = (72/82) 87.8% AT = (10/82) 12.2% TT = 0%	A = 0.93 T = 0.06	0.325	0.00706 (East Asian)
2	c.250T>C	p.(Ser84Pro)	rs1470227348	64577332	Likely pathogenic	TT = (80/82) 97.5% CT = (2/82) 2.4% CC = 0%	T = 0.987 C = 0.012	0.00000417	Not available for Asian population
3	c.249_252delGTCT	p.(Ile85SerfsTer33)	rs587776841	64577330	Pathogenic	1.12%	–	Data not available*	
4	c.892_893delinsTA	p.(Pro298Tyr)	Yes	64574517	Likely pathogenic	1.12%	–	Not applicable[&]	
5	c.856G>T	p.(Gly286Trp)	Yes	64574554	Likely pathogenic	GG = (80/82) 97.5% GT = (2/82) 2.4% TT = 0%	G = 0.987 T = 0.012		
6	g.840+1G>T	Splice region[#]	Yes						
7	c.945G>A	Synonymous	Yes	64573823	Likely benign	GG = (81/82) 98.7% GA = (1/82) 1.21% AA = 0%	G = 0.99 A = 0.006		
8	c.967A>G	p.(Ile323Val)	rs1565644220	64573801	Likely pathogenic	AA = (81/82) 98.7% AG = (1/82) 1.12% GG = 0%	A = 0.99 T = 0.006	Data not available*	
9	c.1075T>G	p.(Cys359Gly)	Yes	64573232	Uncertain significance	TT = (81/82) 98.7% GT = (1/82) 1.12% GG = 0%	T = 0.99 G = 0.006	Not applicable[&]	
10	c.1121delA	p.(Asn374Metfs*8)	Yes	64573186	Pathogenic	1.12%	–		
11	c.1506G>C	p.(Lys502Asn)	Yes	64572148	Uncertain significance	GG = (81/82) 98.7% CG = (1/82) 1.12% CC = 0%	G = 0.99 C = 0.006		
12	c.1838A>G	p.(Lys613Arg)	Yes	64571816	Uncertain significance	AA = (79/82) 96.3% GA = (3/82) 3.6% GG = 0%	A = 0.981 G = 0.018		
	Variants								
13	c.1785G>A	Synonymous	Yes	64571869	Likely benign	GG = (80/82) 97.5% AG = (2/82) 2.4% AA = 0%	G = 0.987 A = 0.012	Not applicable[&]	
14	c.1817C>T	p.(Ser606Phe)	Yes	64571837	Uncertain significance	CC = (79/82) 96.3% TC = (3/82) 3.6% TT = 0%	C = 0.981 T = 0.018		
15	c.1845C>A	synonymous	Yes	64571809	Likely benign	CC = (1/82) 98.7% AC = (1/82) 1.12% AA = 0%	C = 0.99 A = 0.006		

(Continues)

Table 1. Continued

Splice region	Germline variant/ variant	Protein change	Novel	Loci number	Pathogenicity	Genotypic frequency**	Allelic frequency**	gnomAD allele frequency (overall) (Asian)
16	c.1525C>A	p.(Leu509Met)	Yes	64572129	Uncertain significance	CC = (79/82) 96.3% AC = (3/82) 3.6% AA = 0%	C = 0.98 A = 0.018	
17	c.1601C>G	p.(Pro534Arg)	Yes	64572053	Uncertain significance	CC = (81/82) 98.7% GC = (1/82) 1.12% GG = 0%	C = 0.99 G = 0.006	
18	c.1566G>A	Synonymous	rs1367560811	64572088	Likely benign	GG = (81/82) 98.7% AG = (1/82) 1.12% AA = 0%	G = 0.99 A = 0.006	Data not available[†]
19	c.1569T>C	Synonymous	Yes	64572085	Likely benign	TT = (81/82) 98.7% CT = (1/82) 1.12% CC = 0%	T = 0.99 C = 0.006	Not applicable[&]
20	c.1475C>G	p.(Ser492Cys)	Yes	64572179	Uncertain significance	CC = (81/82) 98.7% GC = (1/82) 1.12% GG = 0%	G = 0.99 C = 0.006	
21	c.1581C>G	Synonymous	Yes	64572073	Likely benign	CC = (81/82) 98.7% GC = (1/82) 1.12% GG = 0%	C = 0.99 G = 0.006	
22	c.1511C>T	p.(Ala504Val)	Yes	64572143	Uncertain significance	CC = (81/82) 98.7% TC = (1/82) 1.12% TT = 0%	C = 0.99 T = 0.006	
23	c.1581C>T	Synonymous	Yes	64572073	Likely benign	CC = (81/82) 98.7% TC = (1/82) 1.12% TT = 0%	C = 0.99 T = 0.006	
24	c.1730C>T	p.(Ser577Leu)	Yes	64571924	Likely pathogenic	CC = (81/82) 98.7% TC = (1/82) 1.12% TT = 0%	C = 0.99 T = 0.006	
25	c.1520delA	p.(Lys507Argfs*57)	Yes	64572134	Pathogenic	1.12%	–	
26	c.1630G>C	p.(Ala544Pro)	Yes	64572024	Uncertain significance	GG = (81/82) 98.7% CG = (1/82) 1.12% CC = (1/82) 1.12%	G = 0.99 C = 0.006	

HWE = Hardy-Weinberg Equilibrium.
[#]Splice region mutation was analyzed using RNAsnp tool. It is described separately.
[$]5′UTR variation was analyzed using RNASNP and RegRNA 2.0. Few variations were co-occurring in multiple subjects.
[†]GnomAD has indicated that the data quality is suspect with code AC0 and RF.
[&]Not applicable for novel variants.
*These variants do not have gnomAD exomes entries, but their loci are covered in gnomAD exomes.
**Calculated genotypic and allelic frequency of single base substitution. All variants were having HWE in range on 0.97–0.99.

patients). In addition, we recruited 12 age- and sex-matched healthy controls. The complete follow-up to 36 months (median) were recorded for all patients after discovering PHPT.

Demographic, clinical, and biochemical features sporadic PHPT subjects

The mean age of the patients ($n = 82$) was 41.7 ± 15.3 years (range: 12–65 years) with a female to male ratio 2:1 (54 women and 28 men); 73 patients (89%) were symptomatic. Baseline clinical and biochemical parameters are shown in Table S2.

Germline variants of *MEN1* gene

Of the 82 sporadic PHPT patients, 42 (51.2%) patients showed 26 different germline *MEN1* variations. Out of 26 variants, 21 were novel variants. Of these 26 germline variants, the highest frequencies of variants were found in exon 10 of *MEN1* (Fig. 2). About 61.5% (16/26) of the variations were identified in exon 10. Among 42 patients with 26 different *MEN1* variations, 26.9% (7/26) variants were synonymous, 50% (13/26) were nonsynonymous missense variations, 15.3% (4/26) were deletion variations, and two (c.−35A>T and g.840+1G>T) were found in the noncoding regions in the 5′UTR of exon 1 and splice region of exon 6, respectively. Based on genotype frequency, c.1838A>G, c.1817C>T, c.1525C>A, c.−35A>T (5′UTR), c.250T>C, and c.856G>T were the most common germline variants among the nonfamilial PHPT.

Prediction of pathogenic, benign (nonpathogenic), and uncertain clinical significance nature of variants using in the silico–based approach

Among 26 germline variants, eight (34.7%) were considered pathogenic, seven variants (26.9%) were benign, and the remaining nine (34.6%) were labeled as VUS (Table 1). We explored multiple databases, including VarSome. VUS showed variable scores in different prediction bioinformatics tools under VarSome, so there is uncertainty in determining if these variants were normal or disease-causing. g.840+1G>T was predicted to be splice site pathogenic variation by the EX-SKIP tool; g.840+1G>T had a higher probability of exon skipping than the wild-type allele. The gnomAD allelic frequencies of all the variants were investigated. However, only c.−35A>T variant was found to have both worldwide and East Asian gnomAD allelic frequencies of 0.325 and 0.0076, respectively (Table 1). The predicted ratio of the exon-splicing site and exon splicing enhancer was mentioned in Table S3. The higher ratio exonic splicing silencer /exonic splicing enhancer (ESS/ESE) (0.50) of mutant as compared to the wild-type ratio (0.47) is predicted to be associated with a higher chance of exon skipping in g.840+1G>T, and is thus considered pathogenic.

Menin structure-based functional analysis of pathogenic nonsynonymous variants

Using the UCSF Chimera program, we performed the structural-based functional analysis of frequently identified *MEN1* variants; p.(Gly286Trp), p.(Ser84Pro), and p.(Ser606Phe). Results indicated that the significant atomic contacts/steric clashes of the mutated amino acids with their adjacent side residue directly affect the protein stability. The p.(Ser84Pro) revealed that wild-type amino acid serine is polar, and substituted proline is hydrophobic, directly affecting menin stability. Further, we observed two rotamers of p.(Ser84Pro), out of which the highest one (probability 99%) had two clashes with Asp82, and other rotamers with a probability of 9% had three clashes with Asp82 (Fig. 3*A*–*C*). In p.(Ser606Phe), substituted phenylalanine rotamer (probability 36.2%) had three clashes with Asp602, causing structural alterations in menin (Fig. 3D,E). However, no significant steric clashes were observed in the case of p.(Gly286Trp).

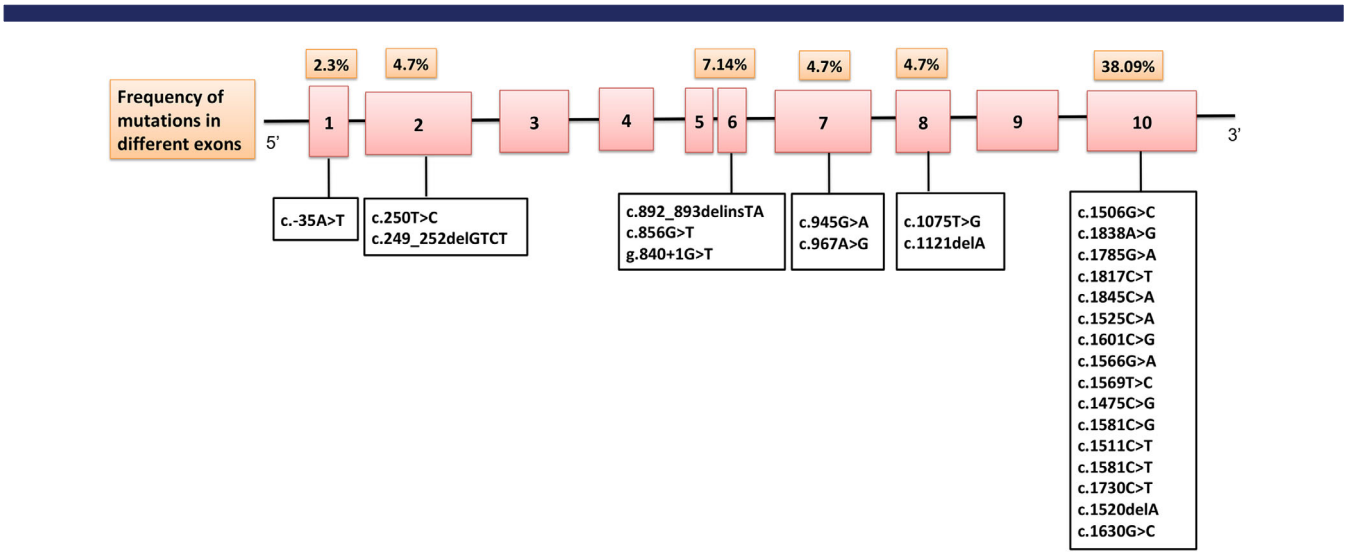

Fig. 2. A schematic representation of the genomic organization of the *MEN1* gene including the germline variants (pathogenic, synonymous, and VUS) and their frequency in different exons identified in our study. The frequency of mutations has been calculated as ratio of number of variations in an exon out of total number of PHPT subjects with *germline* variations ($n = 42$). PHPT = primary hyperparathyroidism; VUS = variant of unknown significance.

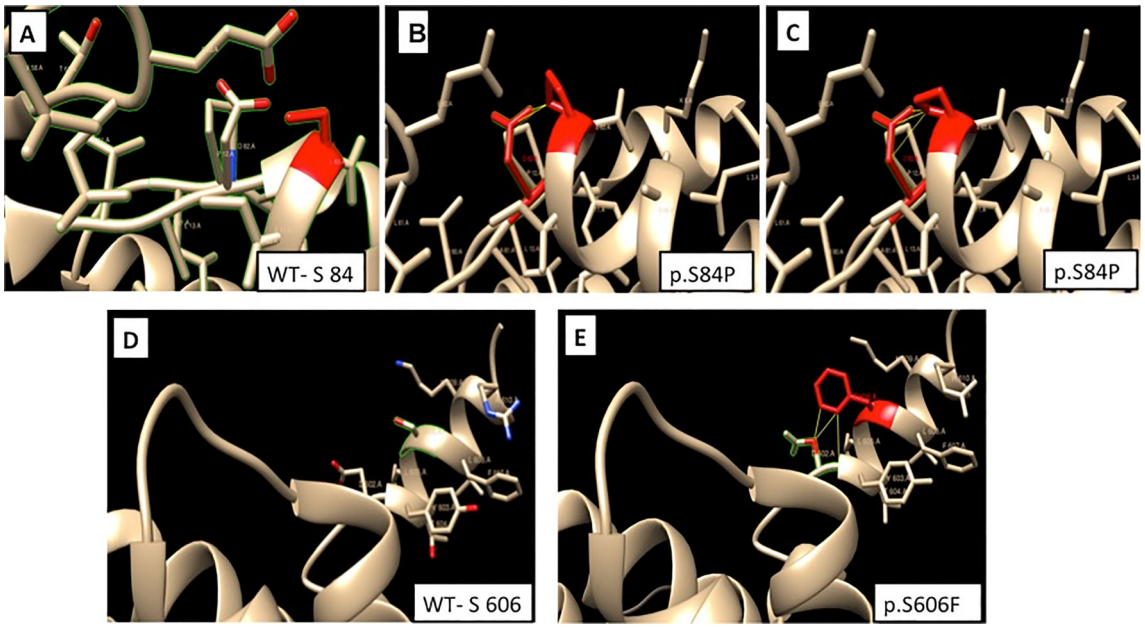

Fig. 3. Molecular dynamics snapshot showing the effects of the pathogenic variations (p. Ser84Pro and p.Ser606Phe) on the menin protein structure. (*A*) Wild-type of menin showing serine residue at position 84; (*B*) the first rotamer of p.S84P (two clashes); (*C*) the second rotamer of p.S84P (two clash); (*D*) wild-type of menin showing serine residue at position 606; and (*E*) the first rotamer of p.S606F (three clash).

Disease-causing potential of VUS using in silico approach

Combining Align-GVDV, FATHMM, and MutationTaster increased the accuracy of predicting the disease-causing potential of VUS. Align-GVDV was used to assess the functional effect of VUS, with alignments to similar sequences of *Homo sapiens*. Out of nine VUS, five variants occurred at strongly conserved residues with GV = 0 and GD ≥ 65 (Table 2). Thus, these five variants belong to class (C65) of substitutions that are strongly like to impede functions. One variant was most likely interfering with function (C55), an additional two variants had a low GD score (C25), and the remaining one variant was not likely to compromise menin function (C0) (Table 2).

In FATHMM, the disease-specific algorithm was used to rank the VUS according to 17 disease concepts. The disease-specific predictions in FATHMM have been developed to predict the functional consequences of cancer-associated variants. The variants with a higher likelihood of being associated with the disease of interest are ranked higher than those variants that are unlikely to be associated with the disease of interest. A prediction score of less than zero indicates a chance of association of variants with the disease of interest, with lower scores indicating an increase in confidence of association. All nine VUSs lie in the range of (−4.98 to −5.25), indicating a positive association with the development of parathyroid adenoma (Table S4).

MutationTaster, a web-based tool, predicts variants' detailed effect on regulatory regions, genomic regions, amino acid changes, splicing patterns, posttranslational modifications, and Kozak consensus sequence alternations. Table S5 summarizes the effect of nine VUS on histone methylation pattern, loss/gain of splice sites, and posttranslational changes in menin protein.

Disease-causing potential of synonymous variants using in silico approach

Investigating the functional consequences of synonymous variants was of utmost importance for predicting the disease-causing

Table 2. The Prediction Score of Different Combination of VUS Using Align GVGD

Variants	Protein change	Type of mutation	GV	GD	Prediction
c.1075T>G	p. (Lys502Asn)	VUS	0.00	93.88	C65
c.1506G>C	p. (Cys359Gly)	VUS	0.00	158.23	C65
c.1838A>G	p. (Lys613Arg)	VUS	0.00	26.00	C25
c.1817C>T	p. (Ser606Phe)	VUS	0.00	154.81	C65
c.1601C>G	p. (Pro534Arg)	VUS	0.00	102.71	C65
c.1525C>A	p. (Leu509Met)	VUS	0.00	14.30	C0
c.1475C>G	p. (Ser492Cys)	VUS	0.00	111.67	C65
c.1511C>T	p. (Ala504Val)	VUS	0.00	64.43	C55
c.1630G>C	p. (Ala544Pro)	VUS	0.00	26.87	C25

VUS = variants of unknown significance.

Table 3. The Predicted Pathogenic Effects for Synonymous Mutation

Variants	Regulatory features	Splice sites	Protein features	Length of protein	Amino acid change
c.1785G>A	Histone 3 lysine 9 mono-methylation, DNAse 1 hypersensitive site, Histone 3 lysine 4 mono-methylation, Histone 3 lysine 36 tri-methylation, Histone 4 lysine 20 mono-methylation & Histone 2b lysine 5 mono-methylation.	Donor site increased at genomic DNA position 6898. Wt: 0.48/mu: 0.95	Phosphothreonine at 599 amino acid (aa) position might get lost	Normal	No
c.945G>A	Histone 3 lysine 36 tri-methylation, Histone 4 lysine 20 mono-methylation, Histone 2b lysine 5 mono-methylation.	Donor site increased at genomic DNA position 4939. Wt: 0.48/mu:0.85	AA (219–395): Interaction with FANCD2 might get lost. Phosphothreonine at 399, 548, and 599 (aa) position might get lost.	Normal	No
c.1845C>A	Histone 3 lysine 9 mono-methylation, DNAse 1 hypersensitive site, Histone 3 lysine 4 mono-methylation, Histone 3 lysine 36 tri-methylation, Histone 4 lysine 20 mono-methylation, Histone 2b lysine 5 mono-methylation.	Donor site marginally increased at genomic DNA position 6957. Wt: 0.5521/mu: 0.5522	NA	Normal	No
c.1566G>A	Histone 3 lysine 36 tri-methylation, Histone 4 lysine 20 mono-methylation	Donor site increased at genomic DNA position 6675 and 6674 Wt: 0.24/mu: 0.81, mu at 6674: 0.91	Phosphothreonine at 548 and 599 (aa) position might get lost	Normal	No
c.1569T>C	Histone 3 lysine 36 tri-methylation, Histone 4 lysine 20 mono-methylation	Donor site marginally increased at genomic DNA position 6673. Wt: 0.97/mu: 0.98	NA	Normal	No
c.1581C>G	Histone 3 lysine 36 tri-methylation, Histone 4 lysine 20 mono-methylation, CCCTC-binding factor gene associated regulatory factor	Donor site marginally increased at genomic DNA position 6687. Wt: 0.35/mu: 0.41	NA	Normal	No
c.1581C>T	Histone 3 lysine 36 tri-methylation, Histone 4 lysine 20 mono-methylation, CCCTC-binding factor gene associated regulatory factor	Donor site increased at genomic DNA position 6698 Wt: 0.62/mu: 0.72	Phosphothreonine at 548 and 599 (aa) position might get lost	Normal	No

mu = Mutant type; Wt = wild-type.

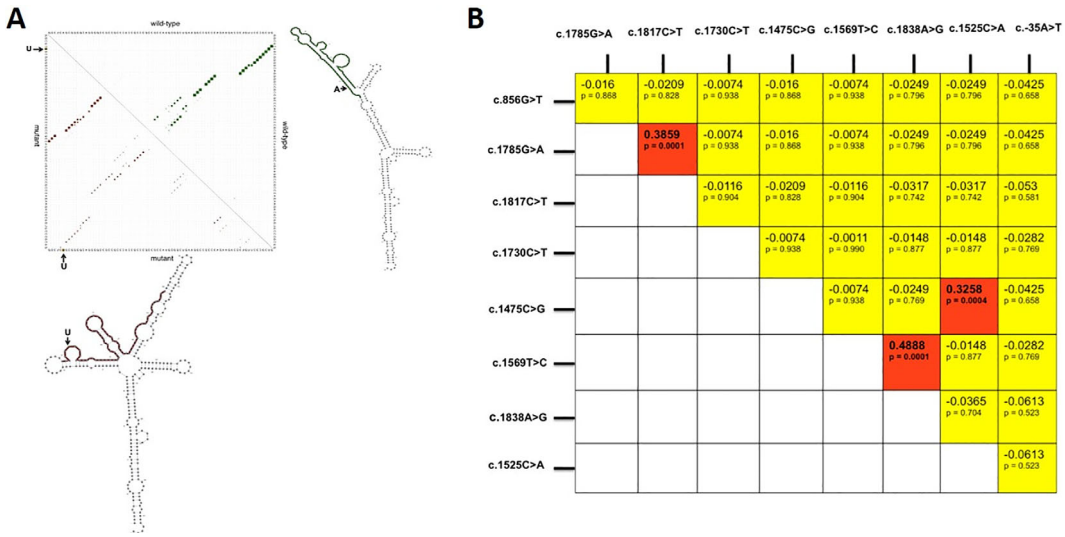

Fig. 4. (*A*) Results of c.−35A>T rs679946 (5′UTR variations) predicted to cause significant local RNA secondary structure changes in 5′UTR of *MEN1* mRNA. (*B*) LD plot for *MEN1* gene variants c.856G>T, c.1785G>A, c.1817C>T, c.1730C>T, c.1475C>G, c.1569T>C, c.1838A>G, and c.1525C>A. LD plot displaying r^2 value and *p* value among variants. Yellow color depicts no significant LD whereas red depicts significant linkage disequilibrium. LD = linkage disequilibrium.

potential. We have employed the MutationTaster tool as it used different analysis models of synonymous and nonsynonymous variants. The detailed functional characteristics of all seven synonymous variants are summarized in Table 3. As per analysis, synonymous variations lead to loss of a phosphothreonine site at amino acid positions 399, 548, and 599 in menin and loss of interaction with Fanconi anemia group D2 (FANCD2) (Table 3).

Prediction and analysis of regulatory variation on RNA secondary structure

The c.−35A>T (5′UTR) was the most frequent (12.1%) variation identified in our cohort. The structural effect was predicted using RNAsnp; we calculated both Euclidean distance (d_{max}) and Pearson correlation coefficient (r_{min}) in mode 1, which was based on the global folding pattern of RNA to predict the effect on local RNA secondary structure. At a significance level of 0.2, the r_{min} and d_{max} were 0.1037 ($p = 0.1$) and 0.2380 ($p = 0.09$) that would predict the structural change in the local region of RNA. The global base pair probabilities of local regions of wild-type and mutant sequences depicted changes in secondary structure with the minimum free energy of −123.50 kcal/mol in wild-type sequence to −120.30 kcal/mol in mutant sequence (Fig. 4*A*). To further characterize the impact of c.−35A>T (rs679946) on the gain or loss of regulatory RNA motifs, we subjected the wild-type and mutant *MEN1* mRNA sequence to the RegRNA tool. We have identified BEN transcriptional regulatory motif (5′-CAGCGGGG-3′) lying within nucleotide positions 75 to 82. This regulatory c.−35A>T SNP results in a BEN transcriptional regulatory motif loss.

Clinical and biochemical comparison between germline *MEN1* PHPT and non-mutant PHPT

A comparison of clinical, biochemical, and postoperative characteristics of 82 sporadic PHPT patients with and without germline *MEN1* variations is summarized in Table 4. Patients with germline *MEN1* variations were younger compared to those without variations ($p = 0.0001$). In addition, serum calcium level was significantly higher in patients with germline *MEN1* variations than those without variations ($p = 0.041$). Further, the comparative analysis of clinical, biochemical, and postoperative characteristics in PHPT subjects with VUS and synonymous (Table S6) was similar to PHPT patients, including pathogenic variants. Among 42 sporadic PHPT with germline *MEN1* mutations, only four subjects were found to have MEN1-related tumors on subsequent screening. All four subjects had microprolactinoma in addition to gastrinoma ($n = 2$) and nonfunctional pancreatic neuroendocrine tumor ($n = 1$).

Phenotypic modulation of polymorphism in the *MEN1* gene in overall PHPT cohort

The c.−35A>T variant was associated with parathyroid adenoma with an OR of 8.09 (95% CI, 0.43–149; $p = 0.094$). The c.−35A>T variant was significantly associated with the subsequent detection of other MEN-related tumors (pituitary adenoma and/or pancreatic neuroendocrine tumors) with an OR of 13.6 (95% CI, 1.1−147; $p = 0.036$) (Table 5). After the first incidence of parathyroid adenoma and parathyroidectomy, three PHPT subjects were diagnosed with multiple endocrine tumors in subsequent follow-up durations. Subject 1 (age 45 years, male) was found to have a microprolactinoma and a pancreatic neuroendocrine tumor (biochemically gastrinoma), subject 2 (age 65 years, male) was found to have a microprolactinoma, and subject 3 (age 50 years, male) also had a pituitary microprolactinoma and a biochemically nonfunctional pancreatic neuroendocrine tumor. We have also calculated the OR of a particular mutant genotype with the recurrence of parathyroid tumors after the surgical intervention (Table 5). c.−35A>T was significantly associated with recurrence of parathyroid tumors with an OR of 5.4 (95% CI, 1.13–25.8; $p = 0.04$) (Table 3). Five subjects with c.−35A>T have shown

Table 4. The Comparison of Clinical, Demographic, Biochemical, Postoperative Parameters and Recurrence Between PHPT With and Without *MEN1* Variations

Parameters	PHPT with germline *MEN1* variations ($n = 42$)	PHPT without germline MEN1 variation ($n = 40$)	p
Clinical characteristics, *n* (%)			
Bone pain[#]	36 (85.7)	30 (71.4)	0.110
Fractures[#]	19 (45.2)	9 (22.5)	0.0003
Renal stone disease[#]	26 (61.9)	16 (40)	0.047
Demographic and biochemical, mean ± SD			
Age (years)	31.1 ± 12.8	53 ± 9.2	0.000
S.Ca (mg/dL) (8.6–10.2)	12.35 ± 1.2	11.7 ± 1.3	0.041
P (mg/dL) (2.5–4.5)	2.4 ± 0.8	2.7 ± 0.9	0.117
ALP (IU/L) (40–129)	412 ± 68*	863 ± 36*	0.090*
iPTH (pg/mL) (15–65)	794.1 ± 124.3*	564.4 ± 111.2*	0.133*
25,dihydroxy vitamin D (ng/mL)	19.5 ± 12	24.1 ± 13.9	0.285
Serum creatinine (mg/dL) (0.5–1.2)	0.94 ± 0.49	1.10 ± 0.9	0.33
Postoperative parameters			
Weight of excised parathyroid adenoma (g), mean ± SE*	2.51 ± 0.4	2.1 ± 0.6	0.667
Recurrence**	10 (23.8%)	0	0.001

Values of p are for the comparison between PHPT with germline MEN1 and no variations group. Data are expressed as mean ± SD or mean ± SE as indicated; *t* test was used for comparison between two groups.

SD = standard deviation; SE = standard error.

*Mean ± SEM.

**Fisher's exact test.

***$p < 0.0005$.

[#]Chi-square test.

recurrent PHPT after single gland parathyroidectomy over the follow-up period of 36 months. c.1525C>A (OR = 13; 95% CI, 1.1–147; $p = 0.03$) mutant genotypes were associated with the presentation of multiglandular parathyroid tumors (Table 5). Three subjects with c.1525C>A has shown multiglandular parathyroid tumors.

LD analysis

The pattern of pairwise LD distributions of c.856G>T, c.1785G>A, c.1817C>T, c.1475C>G, c.1730C>T, c.1569T>C, c.−35A>T, c.1838A>G, c.1525C>A frequently occurring variants of the *MEN1* gene is shown in Fig. 4*B*. Based on *r* statistics values, c.1785G>A and c.1817C>T ($r^2 = 0.3859$, $p = 0.0001$), c.1475C>G and c.1525C>A ($r^2 = 0.385$, $p = 0.0004$), and c.1569T>C and c.1838A>G ($r^2 = 0.488$, $p = 0.0001$) had significant LD. As described (Table S7), the haplotype 1–5, showed the high OR of development of parathyroid adenoma as compared to control haplotype.

Discussion

In the present study, we evaluated different types of *MEN1* germline variants in PHPT. We classified the novel and known variants based on their predicted role of the affected residues, their detailed clinical analysis of associations with PHPT, and investigated their structural and functional consequences on menin function.

The last decade has seen remarkable progress in identifying 1336 germline *MEN1* variations in PHPT.[8,19-22]. However, the exact role of different variations was not investigated apart from identification. Also, most studies lack genotype–phenotype correlation concerning the severity of PHPT that decreases physician-directed monitoring after surgical intervention.[3,23-24] The presentation of PHPT in developing countries like India is primarily symptomatic with an early-age onset, more severe biochemical abnormalities, and higher tumor weight as compared to the western cohorts.[25,26] To the best of our knowledge, no evidence of robust screening, classification, and association of *MEN1* gene variations in symptomatic nonfamilial PHPT has been reported.

Further, the information regarding the disease-causing potential of synonymous, VUS, and pathogenic variants are still lacking. We have integrated the Sanger sequencing of *MEN1*, in silico analysis, clinical association, structural assessment, and haplotype analysis to investigate the relationship of functional consequences, structural abnormalities, and disease-specific potential of *MEN1* variants. This study represents a single-institutional *MEN1* variations analysis in 82 consecutive PHPT patients who underwent surgical removal of parathyroid adenoma. The screening of PHPT for *MEN1* gene variations allows a generalization of our demographic, biochemical, and postoperative findings to the actual situation of its contribution to our population disease severity mechanism.

In this study, we have identified 26 different germline MEN1 variations in 42 sporadic PHPT patients. Previous studies reported a prevalence of 4.6% of *MEN1* germline mutations in 86 clinically nonfamilial sporadic PHPT patients.[27,28] Another study by Uchino and colleagues[29] highlighted the lack of family history in 23% of cases of MEN1 mutation in sporadic PHPT, thereby underestimating the MEN1 prevalence in sporadic PHPT. Variations were mainly found in exons 1, 2, 6, 7, 8, and 10, whereas no variations were observed in exons 3, 4, 5, and 9. The lack of variations in these exons may be a contemplation

Table 5. Genotype–Phenotype Association of *MEN1* Gene Variants With Disease Parameters

Variants	Single-site versus multiple-site tumor				Recurrent versus nonrecurrent PA				Uni-glandular versus multiglandular tumor			
	SST ($n = 38$)	MST ($n = 4$)	OR (95% CI)	p	NR ($n = 32$)	R ($n = 10$)	OR (95% CI)	p	UG ($n = 32$)	MG ($n = 10$)	OR (95% CI)	p
c.856G>T (likely pathogenic)	2	0	–	1.000	2	0	–	1.00	2	0	–	1.000
c.1785G>A (likely benign)	2	0	–	1.000	1	1	3.4 (0.19–6.0)	0.424	2	0	–	1.000
c.1817C>T (uncertain significance)	3	0	–	1.000	3	0	–	1.00	3	0	–	1.000
c.250 T>C (likely pathogenic)	3	0	–	1.000	2	0	–	1.00	2	1	1.7 (0.13–20)	1.000
c.1475C>G (uncertain significance)	2	0	–	1.000	1	1	3.2 (0.19–60)	0.424	1	1	3.4 (0.19–60)	0.424
c.1569T>C (likely benign)	0	1	–	0.095	0	1	–	0.236	0	0	–	0.238
c.1838A>G (uncertain significance)	3	0	3.889	0.341	2	1	3.7 (0.45–30)	0.236	0	1	–	0.238
c.1525C>A (uncertain significance)	3	0		1.000	1	2	3.7 (0.45–30)	0.236	**0**	**3**	**13.2 (1.19–147)**	**0.036**
c.−35A>T (likely benign)	7	3	**13.286 (1.1–147)**	**0.036**	**5**	**5**	**5.4 (1.13–25.8)**	**0.04**	**6**	**4**	**3.6 (0.9–14)**	**0.213**

CI = confidence interval; MG = multiglandular; MST = multiple-site tumor; NR = nonrecurrence; OR = odds ratio; PA = parathyroid adenoma; R = recurrence; SST = single-site tumor; UG = uni-glandular.

of the bounded number of DNA sequence abnormalities identified to date rather than a true "cold" region for mutations. Interestingly, exon 10 harbors 38% of the reported variations, indicating a possible "hot" spot for *MEN1* variations in the Indian PHPT population. The majority of PHPT patients with *MEN1* variations were younger (31.1 ± 12.8 years) compared to non-mutant PHPT in our study. Recent reports embark on comparing age differences among PHPT patients with germline *MEN1* and without mutation.[19] The Italian *MEN1* registry and other studies have estimated that the average age of the first *MEN1* manifestation was 41.6 years, and the average age was 55.1 years,[19,27,30-32] which is one to two decades later than our cohort, predicting the earlier onset of disease in Indians. Recent findings also estimated that 10% of patients with sporadic PHPT under 45 years carry germline *MEN1* variations.[27,28] We observed that the majority of germline *MEN1*-PHPT were symptomatic, with higher serum calcium levels. Similar studies have also reported higher serum calcium levels in *MEN1*-PHPT.[18,29,33-35] We found a significant increase in renal stones disease in patients with germline *MEN1* variations. This finding is consistent with Lourenço and colleagues.[36] With respect to these findings, similar studies have reported higher serum calcium (mg/dL) levels in *MEN1*-PHPT.[26,37,38] We found that bone pain was the common manifestation (87.5%) compared to non-mutant PHPT (71.4%) with a significantly higher fracture rate; a fracture rate consistent with previously reported studies on the *MEN1*-PHPT.[29,34-38] The high fracture rate in *MEN1*-PHPT may be due to the early onset of the disease that might interfere with the achievement of peak bone mass.[37,39] Another reason could be that the mutated *MEN1* might affect menin function, which has a role in bone development.[40]

Our analysis identified eight pathogenic, nine uncertain significance, seven synonymous (benign), based on the score predicted by PolyPhen-2, PROVEAN, and VarSome database.[14-16] Among nine pathogenic variations, c.249_252delGTCT, c.1121delA, c.892_893delinsTA, and c.1520delA were deletion-insertions likely to result in termination of translation and in functional loss of the menin protein.[41] Ser84Pro, Gly86Trp, and Ser606Phe were most frequently occurring variations. Using a computational approach, we assessed the structural changes in the mutant menin and the molecular mechanism of functional impairment by missense *MEN1* variations. The variation at the conserved site mounts the structural changes in protein, affecting the menin stability. Steric clashes in Ser84Pro revealed that wild-type polar amino acid serine to hydrophobic proline substitution directly affected the menin-stability. S606F substitution entails multiple clashes with Asp602, causing considerable structural alterations in menin, negatively affecting structural stability.

The VUS in *MEN1* present a considerable clinical challenge, so to characterize the exact role of VUS, a combination of multiple algorithms, Align-GVGD, FATHMM, and MutationTaster[42] was used. According to Align-GVDV, most VUS (K502N, C359G, S606F, P534R, S492C) belong to the C65 class and are considered to possess disease-causing potential. The advantage of using Align-GVGD is that it provides quantitative measures of the range of biochemical variation of the amino acids at the position of a missense substitution (GV) and the distance between the missense substitutions with a range of variation (GD). One can thus easily track down the characteristics of amino acids and helps to explain the purpose for strong or weak correlations between GV, GD, and function.[43] We also used FATHMM to prioritize VUS functional consequences. The ranking of variants in FATHMM was based on 17 disease concepts with 20-fold cross-

validation, reducing false-positive results. All VUS were shown to have disease-causing potential with a high confidence score in FATHMM analysis. Further, it is imperative to analyze VUS variants' effect on genomic, regulatory, and posttranslational regions of menin. Results from the mutation taster showed that K502N, C359G, S606F, P534R, S492C, A544P, A504V, L509M, and K613R were affecting histone machinery, particularly Histone 3 lysine 36 trimethylation (H3K36me3), Histone 3 lysine 9 monomethylation (H3K9me1), Histone 3 lysine 4 monomethylation (H3K4me1), Histone 4 lysine 20 monomethylation (H4K20me1), and Histone 2b lysine 5 monomethylation (H2bK5me1). The H3K36me3 plays an important role in the dynamic deposition of N6-methyladenosine (m6A) methylation in mRNA via METTL14, affecting gene expression.[44] Hence changes in gene-specific H3K9me1, H3K4me1, H4K20me1, and H2bK5me1 might be affecting MEN1 mRNA expression. Previous studies of colorectal cancer have shown changes in gene-specific histone methylation patterns.[45] The analysis has also predicted the loss of alpha helix in K613R, S606F, A504V, and A544P in menin. Previous biochemical studies have shown that alpha helix is essential for the interaction of menin with MLL1 and LEDGF.[46] Various studies have highlighted the importance of synonymous variations as they play a fundamental role in multiple diseases and might correlate with clinical outcomes.[47] The analysis of MutationTaster has predicted the results of the synonymous variations in *MEN1*-specific alternations in histone machinery. In previous studies, synonymous variations have been associated with splicing regulation.[48] Our study has shown changes in splicing patterns due to synonymous variations (c.1785G>A, c.945G>A, c.1566G>A, and c.1581C>T). Synonymous variations were also shown to affect the posttranslational modifications of menin. c.1785G>A, c.1566G>A, c.1581C>T, and c.945G>A results in loss of phosphorylation of threonine at amino acid position 399, 548, and 599 in menin. c.945G>A also results in loss of interaction with FANCD2. Previous studies show menin–FANCD2 interaction plays an important role in DNA repair (References 6 and 7).

These variations might affect the interaction of menin with multiple transcription factors, which is essential for cellular differentiation and development.[6] S606F is essential for interaction with ASK, CHES1, and Smad3.[49-51] ASK, a component of the Cdc7/ASK kinase complex, plays a crucial role in DNA replication through minichromosome maintenance (MCM) complex and Ask-menin complex, regulating cell proliferation suppression.[51,52] Ches1, a forkhead transcription factor, restrains the DNA damage sensitivity.[53] Menin interacts with CHES1 through its COOH terminus (428–610) and plays a regulatory role in the S-phase checkpoint pathway associated with DNA damage response.[54] Menin interacts with the transforming growth factor β (TGF-β) through Smad3 and amelioration of menin due to mutation impedes Smad3 binding to DNA and blocking TGF-β signaling disturbing the exquisite balanced cellular state, presuming the cell toward tumor formation.[55] Although missense variations G286W do not alter structural instability in terms of steric clashes, this might affect the functional impairment of menin as it occurs in a region that is shown to interact with the placenta and embryonic expression, a subunit of RPA2, HDAC1 and nonmuscle myosin type II-A heavy chain (NMHC II-A).

All affected patients displayed the most frequently occurring variations (c.856G>T, c.1785G>A, c.1817C>T, c.1475C>G, c.1730C>T, c.1569T>C, c.−35A>T, c.1838A>G, and c.1525C>A) were analyzed for its association with the development of parathyroid adenoma, prognosis, and occurrence of multiple endocrine tumors. Compared to the Western world, the Indian PHPT registry from our group[32] suggests that PHPT patients in India manifest severe symptomatic disorders at a younger age.[56,57] Our genotype–phenotype study indicates a possible mechanism behind the severe form of PHPT in India. Our data revealed that c.1525C>A, c.−35A>T, c.1838A>G, and c.250T>C can influence the postoperative outcome of PHPT. However, no evidence about the association of variation with the presence of multiglandular parathyroid adenoma has been established so far. c.1525C>A was significantly associated with the development of multiglandular parathyroid adenoma. Notably, studies have shown multiglandular parathyroid adenoma and its recurrence rate were higher in patients with *MEN1* variations.[37-39] The importance of studying LD in the present study entails if there is a nonrandom co-occurrence of different variants, which could be notified as parathyroid adenoma susceptibility variants.[58] The c.1785G>A and c.1817C>T, c.1475C>G and c.1525C>A, and c.1569T>C and c.1838A>G were significantly showing strong LD to each other, ie, displaying non-random association of co-occurrence of these variants in our patient cohort. Earlier studies did not report any LD among previously reported variations in *MEN1*.[49] So, this is the first study to establish the LD among these novel variants of *MEN1*, which could specify them as parathyroid adenoma susceptibility variants. However, it further requires testing and validation in a larger Indian PHPT population.

A 5′UTR variation (c.−35A>T) was the most frequently (12.1%) occurring variant in our cohort. Though reported earlier,[59-61] its functional association with the disease was not clearly studied. The gnomAD database shows that c.−35A>T has worldwide and East Asian gnomAD allelic frequencies of 0.325 and 0.0076, respectively. However, its clinical relevance in sporadic parathyroid tumors was not thoroughly elucidated. The present study identified a novel association of c.−35A>T with parathyroid adenoma and other endocrine tumors, multiglandular adenoma, and postoperative prognosis. Regulatory variations in the UTR region affect the mRNA secondary structure resulting in aberrant gene regulation.[62,63] The predicted modification in the secondary structure of *MEN1* mRNA might alter the stability of the BEN motif that is involved in the binding of the respective transcription factors.[64,65] BEN is a sequence-specific transcriptional repressor regulating neurogenesis and chromatin functioning.[65,66] Based on our findings, we have predicted the hypothetical model about the mechanism of c.−35A>T, in which c.−35A>T might affect the transcriptional activity mediated by the altered binding behavior of *trans*-acting protein factors to disrupting the BEN domain in the *MEN1* promoter region which affect the proliferation of multiple endocrine cells (Fig. 5), although experimental validation of this model is needed to confirm our findings. c.−35A>T gains importance in PHPT due to its significant association with the development of multiglandular multiple organ tumors (pancreatic neuroendocrine tumors and pituitary adenoma) as patients presenting c.−35A>T presented with pancreatic neuroendocrine tumors and/or pituitary adenoma) and with the recurrence of parathyroid tumors. Therefore c.−35A>T can be a potential predictor and a prognostic genetic marker for PHPT phenotypes and the development of other endocrine tumors.

Limitations

Our study lacks a larger sample size allowing us to better interpret the genotype–phenotype association and haplotype risk assessment among variants of *MEN1*. Further, this study lacks an appropriate sample size of healthy controls of Indian cohort

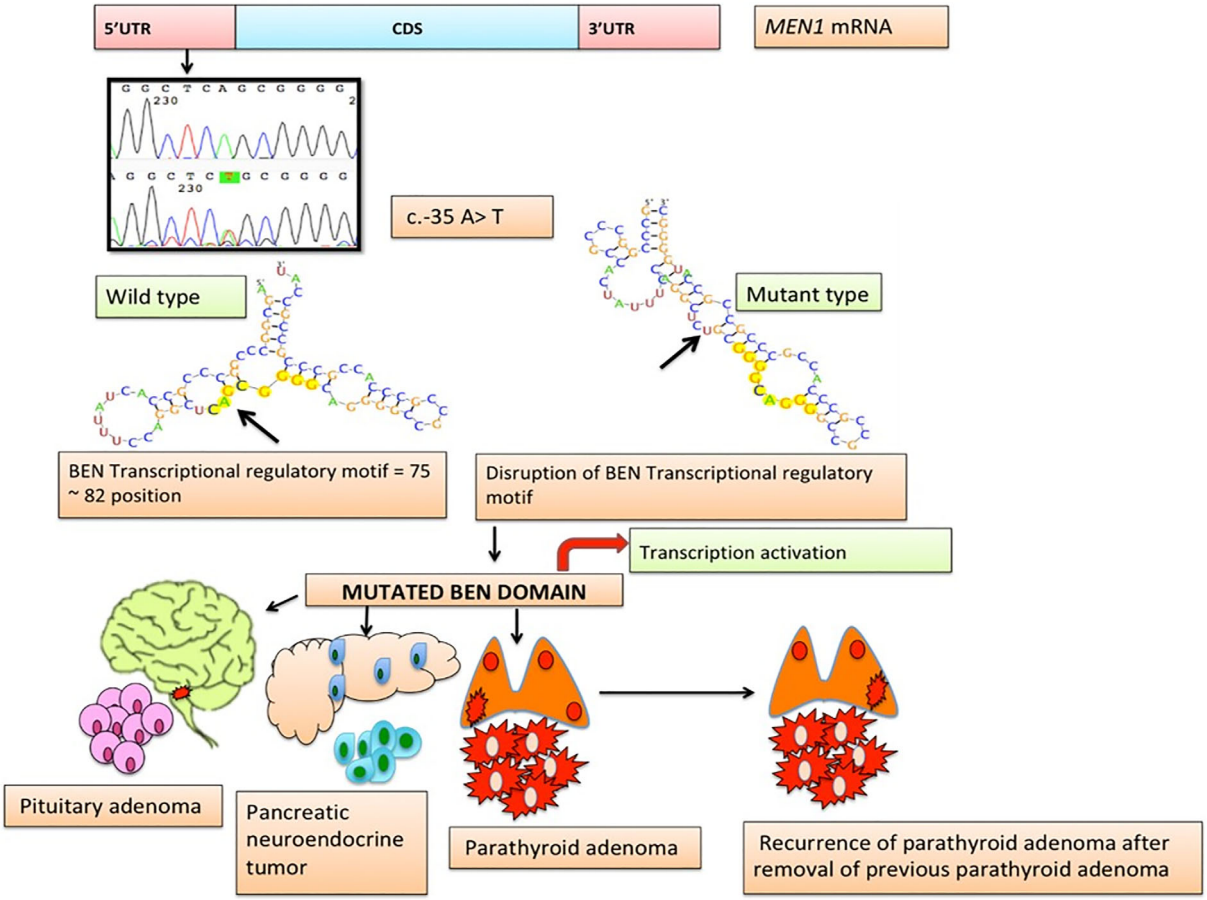

Fig. 5. A schematic representation of the proposed mechanism of c.−35A>T in the development of parathyroid adenoma and its recurrence, pancreatic neuroendocrine tumor and pituitary adenoma via alternation in BEN domain.

for the better validity of *MEN1* variations. Other novel *MEN1* variations, including the c.−35A>T regulatory variant, could be experimentally characterized to better understand the clinical manifestation and the disease pathogenesis. Additional functional studies are needed to fully understand the role of VUS, synonymous and nonsynonymous variants.

Conclusion

The present study highlights the importance of utilizing multiple approaches to investigate the role and contribution of pathogenic, VUS, synonymous, and 5′UTR variations in clinically nonfamilial Indian PHPT patients. Such high frequency can be explained based on the early age onset of PHPT in Asian-Indians, thereby implying a high ethnic-specific prevalence. Furthermore, selective screening of *MEN1* variations, especially those with disease-specific potential, can prompt early screening for other *MEN1*-related tumors in the proband, recurrent and multiglandular parathyroid disease. Besides, we have demonstrated that PHPT patients with *MEN1* variations have an earlier onset of the disease, higher serum calcium, and higher fracture rate. So our observations provide considerable insight into the important aspects of disease-specific variations that might contribute to PHPT and disease expression.

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Author Contributions

Gurjeet Kaur: Conceptualization; data curation; formal analysis; investigation; methodology; software; validation; visualization; writing – original draft; writing – review and editing. **Sanjay Kumar Bhadada:** Data curation; formal analysis; funding acquisition; investigation; project administration; resources; software; supervision; validation; writing – original draft; writing – review and editing. **Mithun Santra:** Data curation; formal analysis; software; validation; visualization; writing – review and editing. **Rimesh Pal:** Data curation; formal analysis; validation; writing – review and editing. **Phulen Sarma:** Data curation; formal analysis; software; writing – review and editing. **Naresh Sachdeva:** Investigation; methodology; resources; supervision; writing – review and editing. **Vandana Dhiman:** Investigation; methodology; visualization; writing – review and editing. **Divya Dahiya:**

Resources. **Uma Nahar Saikia:** Software; supervision; validation; visualization. **Anuradha Chakraborty:** Data curation; formal analysis; validation. **Ashwani Sood:** Investigation; resources; software; visualization. **Mahesh Prakash:** Software; validation. **Arunanshu Behera:** Resources; writing – review and editing. **D. Sudhaker Rao:** Data curation; formal analysis; investigation; writing – review and editing.

Conflicts of Interest

No potential conflicts of interest were disclosed.

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Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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