Original Article

Novel multiple endocrine neoplasia type 1 variations in patients with sporadic primary hyperparathyroidism

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ABSTRACT

Background and Objectives: Primary hyperparathyroidism (PHPT) can occur either as a sporadic case or in association with syndromes such as multiple endocrine neoplasia. Multiple endocrine neoplasia type 1 (*MEN1*) is a rare autosomal-dominant disease resulting from mutations in *MEN1* gene encoding a 621 amino acid long tumor suppressor protein "menin." We report here the results of *MEN1* screening in 31 patients diagnosed with sporadic PHPT. **Materials and Methods:** Diagnosis of sporadic PHPT was made when blood urea and serum creatinine were normal, serum parathyroid hormone was high, and parathyroid enlargement could be localized on ultrasound and/or parathyroid scan. A total of 31 patients and 50 healthy volunteers were recruited for molecular analysis after taking informed consent. **Results:** Major symptoms at presentation were bone pain, fatigue, muscle weakness, and renal stones. Molecular genetic analysis revealed the presence of two novel intronic variations, c. 913-79T>A and c. 784-129T>A which by human splicing finder are predicted to cause potential alteration of splicing by either activating an intronic cryptic acceptor site or converting a conserved exonic splicing silencer sequence to an exonic splicing enhancer site. Apart from these, two reported polymorphisms rs144677807 and rs669976 were seen only in patients and none of the controls. Other reported polymorphisms rs2071313 and rs654440 were identified both in controls and patients. **Conclusions:** This is the first study of *MEN1* gene screening in sporadic PHPT in India reporting on the clinical and genetic findings, wherein two novel intronic variations c. 913-79T>A and c. 784-129T>A were identified showing their possible role in disease causation.

Key words: Multiple endocrine neoplasia type 1 gene, mutations, primary hyperparathyroidism

Introduction

Primary hyperparathyroidism (PHPT) results from hyperfunctioning of the parathyroid glands secreting excessive parathyroid hormone (PTH) and disrupting calcium homeostasis.

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PHPT can be either sporadic or occur in association with genetic syndromes such as multiple endocrine neoplasia-1 and 2 (MEN1 and 2). MEN1 is a rare autosomal-dominant disease characterized by the collective manifestations of tumors in the pancreas, parathyroid, and pituitary. In 90% of the MEN1 cases, PHPT occurs as the first and most common manifestation.[1] MEN1 results from mutations in the *MEN1* (OMIM 613733) gene which encodes a 621 amino acid tumor suppressor protein "menin."[2] We

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report here the results of *MEN1* screening in 31 patients diagnosed with sporadic PHPT.

Materials and Methods

The study protocol was approved by the Institute Ethics Committee. A total of 31 consecutive patients who presented from June 2013 to June 2015 diagnosed with PHPT and who did not show any feature of pituitary or pancreatic tumors were recruited from the Department of Endocrinology and Metabolism. Patients who presented with a history of recurrent renal stones, bone pain, fracture, or those who were referred for evaluation of hypercalcemia underwent biochemical evaluation for serum calcium, phosphorus, albumin, alkaline phosphatase, blood urea, serum creatinine, PTH, 25 (OH) Vitamin D, 24 h urinary calcium, and creatinine. If serum PTH levels were found to be raised with serum calcium levels being normal or raised, the patients underwent an ultrasound of the neck, followed by MIBI nuclear scan of the parathyroid gland to localize cause of hyperparathyroidism. *MEN1* syndrome was ruled out by excluding pituitary and pancreatic involvement on history. Serum fasting levels of prolactin, insulin-like growth factor-1, T4, thyroid-stimulating hormone, luteinizing hormone, follicle-stimulating hormone, prolactin, and adrenocorticotropic hormone were measured to rule out pituitary involvement. PTH was analyzed using electrochemiluminescence assay by Roche. A diagnosis of PHPT was made when blood urea and serum creatinine were normal, serum PTH was high, and parathyroid gland enlargement was found on ultrasound and/or parathyroid scan.

Detailed family history was taken and peripheral blood was drawn from the patients after taking informed consent. Fifty healthy volunteers from hospital staff and patients' spouses formed the control group.

Mutation analysis

Genomic DNA was isolated from peripheral blood leukocytes of the 31 patients and 50 controls recruited using standard protocol and subjected to polymerase chain reaction amplification of all the coding exons of the *MEN1* gene using 80–100 ng DNA, 1.25 mM $MgCl_2$, 0.25 mM of each of the dNTPs (Fermentas, Massachusetts, USA), 20 pM of each primer, and 0.5 units of Taq polymerase (Invitrogen, Carlsbad, CA, USA) in a 25 μl volume mixture using thermocycler ABI 9700 (Applied Biosystems, Foster City, CA, USA).[3] All the amplified products were purified using Qiagen kits (Qiagen, GmbH, Hilden, Germany) and were then sequenced using ABI-3730XL genetic analyzer (ABI). Nucleotide sequences were compared with the published cDNA sequences of *MEN1* gene (GenBank accession number ENST00000312049). Prediction of functional effects of the novel variations was done using algorithms human splice finder (HSF) matrices.[4]

Results

Out of 31 patients, 20 were female and 11 were male, with mean age at presentation of 35.8 ± 13.5 years. There was no history of consanguinity or positive family history of similar illness in any of the patients. Most patients presented with symptoms such as bone pain, fatigue, renal stones, muscle weakness, or abdominal pain [Table 1]. All the patients underwent surgery for removal of the adenoma.

Molecular genetic analysis revealed presence of two novel intronic variations, c. 913-79T>A in intron 6 and c. 784-129T>A in intron 4 in three and one patients, respectively, and in none of the controls [Figure 1].

The intronic variation c. 913-79T>A was predicted by HSF to cause potential alteration of splicing by activation of an intronic cryptic acceptor site. This variation was seen in three patients who were comparatively young (mean age 24 ± 8 years) and presented with common features such as fractures, muscle weakness, and renal colic and

Table 1: Clinical and biochemical characteristics of the patients

Clinical details	Number of patients (*n*=31)
Female/male	20/11
Age (years)	
<50	27
>50	4
Serum calcium (8.5-10.2 mg/dl)	
≤10.2	3
10.2-15	13
>15	15
Serum Vitamin D (ng/ml)	
<10 – deficient	21
10-30 – insufficient	9
30-100 – sufficient	1
Adenoma	
One	28
Two	3
Site of adenomas	
Left inferior	10
Right inferior	16
Left superior	4
Right superior	4
Symptoms, *n* (%)	
Bone pain	24 (77.4)
Fatigue	21 (67.7)
Proximal muscle weakness	20 (64.5)
Renal stones	17 (54.8)
Abdominal pain	15 (48.4)
Fractures	8 (25.8)

gastrointestinal problems. One patient had additional neuropsychiatric symptoms of ataxia and abnormal behavior. Brown tumor was present in two while all three patients with this variation showed the presence of a single adenoma in the right inferior parathyroid gland.

The intronic variation c. 784-129T>A was also predicted by HSF to be pathogenic due to formation of a new exonic splicing enhancer (ESE) site which may lead to mRNA splicing alteration. This variation was identified in a 24-year-old male who presented with a history of abdominal pain. On investigations, he was diagnosed with sporadic PHPT and had one adenoma located in the left superior parathyroid gland.

Apart from this, reported polymorphisms rs144677807 and rs669976 were identified in one and three patients, respectively, and none of the controls. Three reported single nucleotide polymorphisms rs2071313, rs654440, and rs669976 were also identified both in patients and controls [Table 2].

Discussion

Hyperparathyroidism is the most common feature of MEN1 syndrome, followed by pancreatic neuroendocrine and pituitary tumors.[5]

MEN1 syndrome is known to be associated with mutation in *MEN1,* a tumor suppressor gene located on chromosome 11q13, encoding a 621 amino acid protein "menin."[4] It is also one of the genes with an established role in pathogenesis of sporadic parathyroid adenomatosis. Specific clonal alterations involving somatic mutation and/or deletion of both *MEN1* alleles have been demonstrated in about 15–20% of sporadic parathyroid adenomas.[6]

In the present study, we report the clinical findings and *MEN1* gene variations in 31 consecutive patients diagnosed with sporadic PHPT in Indian population.

The mean age of patients in our study was 35 ± 13.5 years and was comparable to previous Indian studies.[7-10] Majority (27/31) of our patients were diagnosed with hyperparathyroidism (HPTH) at age <50 years which is lower than the age reported from developed countries (55–65 years).[11-16] In a retrospective study of PHPT patients from a tertiary care center in North India, majority patients were <50 years old,[10] which is in concordance with our study.

Table 2: *MEN1* gene variations identified in the patients with hyperparathyroidism

Variation	Exon/intron	Patients (*n*=31) (%)	Controls (*n*=50) (%)
c.913-79T>A	Intron 6	3 (9.6)	None (0)
c.784-129T>A	Intron 4	1 (3.2)	None (0)
A237A (rs144677807)	Exon 4	1 (3.2)	None (0)
rs669976	Intron 6	7 (22.5)	None (0)
rs2071313	Exon 9	17 (54.8)	36 (80)
rs654440	Exon 9	8 (25.8)	28 (62)

Most sporadic PHPT adenomas are benign with malignant carcinomas representing about 1% of the cases.[17] None of the patients in the present study had parathyroid carcinoma. In a previous study of 39 patients of PHPT from our center,[9] only two were reported to have parathyroid carcinoma while rest were adenomas.

Severe hypercalcemia (serum calcium >15 μg/dl) was present in nearly half of the patients (15/31) even though majority (30/31) were Vitamin D deficient or had insufficient Vitamin D levels. Symptoms of bone pain were present in majority of the cases which is in concordance with a previous study of PHPT from our center[9] where the mean serum calcium was in hypercalcemic range, i.e., 12.52 ± 1.58 mg/dl (normal: 8.5–10.2 mg/dl), yet the mean Vitamin D was in insufficient range, i.e. 10.21 ± 5.82 ng/ml.

In MEN1 syndrome, PHPT manifests at an earlier age starting from the second decade of life compared to sporadic PHPT where it manifests later.[11] It is recommended that *MEN1* mutation analysis should be carried out in the patients having a single apparently sporadic MEN1-associated tumor presenting at a younger age[18] as several nonsense, missense, frameshift, in-frame deletions/insertions, and splice-site mutations in *MEN1* are known to be associated with MEN1 syndrome.[19,20]

Coding region mutations are known to cause defects in the encoded proteins by changing conserved amino acids. Recently, various studies report on exonic as well as intronic mutations exerting their effects by altering pre-mRNA splicing. It is estimated that up to 60% of disease-causing mutations affect mRNA splicing.[21,22]

mRNA splicing is a crucial mechanism of gene regulation which is mediated by the spliceosome (a multicomponent splicing complex) which forms a network with specific proteins, such as small nuclear ribonucleoprotein (snRNP) and serine/arginine-rich proteins. These components of the splicing machinery recognize precise splice-site sequences and cut the DNA at specific sites resulting in accurate elimination of introns to form the mature mRNA. The specific sequences form the 5' and 3' splice-site sequences, branch point sequence, polypyrimidine tract, intronic splicing enhancer/intronic splicing silencer, and exonic

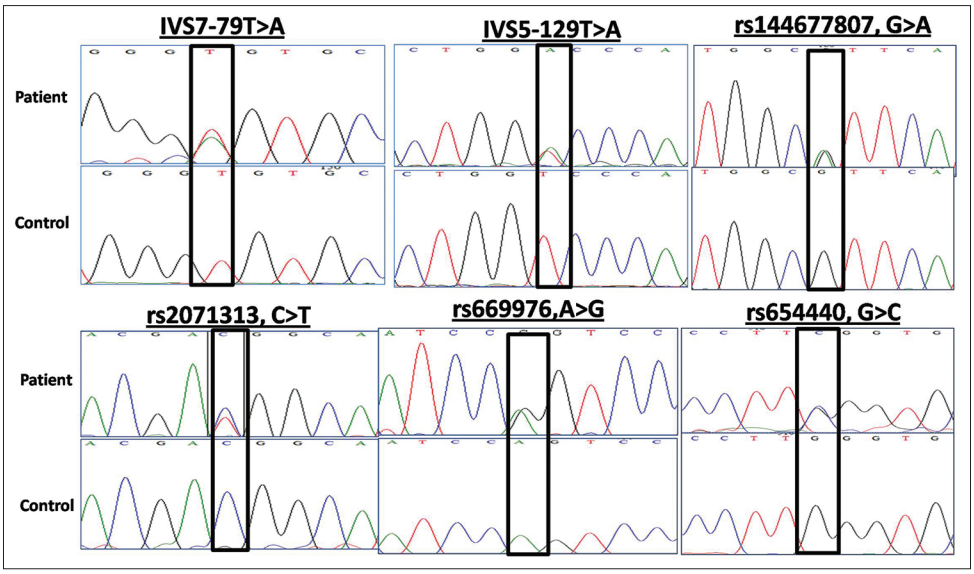

Figure 1: Electropherograms showing different variations found in the present study

splicing silencer/enhancer (ESS/ESE) and play important roles in excision of the introns.[23]

These elements are essential for correct splice-site identification to which the factors such as U1 snRNP, SF1/mBBP, U2 snRNP, U2 snRNP auxiliary factor 65 (U2AF65), and U2 snRNP auxiliary factor 35 (U2AF35) come and bind.[18] In-silico algorithms can predict the possible outcome of intronic mutations on splicing.

Similarly, in MEN1 syndrome, about 5–10% of the patients may not possess an identified *MEN1* coding region mutation rather than they might have mutations in the promoter or the untranslated regions (5' and 3' end).[4]

Mutations affecting splicing can be categorized into various types depending on their position and effect on the splicing pattern. Some of them are single nucleotide substitutions in the conventional sequences such as acceptor or donor splice-site sequences that totally abolish exon recognition. They result in complete skipping of an exon, retention of the mutated intron, or truncated protein (if the intron is too large making it unstable which will not be retained). In addition, they can also result in activation of an upstream or downstream cryptic site. Other mutations occurring within an exon or intron can introduce a new splice site known a cryptic splice site at the preexisting pseudo 5' or 3' splice-site present in proximity.[24] These cryptic donor or acceptor sites lead to alteration in splicing and thereby resulting in defective protein products.

In the present study, the two novel intronic variations c.913-79T>A and c. 784-129T>A identified were predicted to affect splicing and be possibly pathogenic by HSF algorithm.[4]

However, polymorphisms rs144677807 and rs669976 were predicted to have no significant impact on the protein though found only in patients. Polymorphisms rs2071313 and rs654440 found both in patients and controls did not impact the protein and thus, may be normal variations.

Conclusion

This is the first report of *MEN1* gene screening in sporadic PHPT in India. The study reports on clinical and genetic findings of 31 patients with sporadic HPTH, wherein two novel intronic variations c. 913-79T>A and c. 784-129T>A were identified showing a possible association with the disease but needs further validation on a larger sample size.

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Conflicts of interest

There are no conflicts of interest.

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