Sequencing And Site Directed Mutagenesis

Role in analysis of human diseases

For PG Sem III

By

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The prevalence of genetic disorders in India is very high owing to a very large population, high birth rate and the practice of consanguineous marriage in many communities.

Due to inadequate diagnostic, management and rehabilitation facilities, the burden of these disorders is greater than in any western countries.

To overcome this burden on our society genetic analysis, testing and counseling deserves one of the highest priorities in our health curriculum.

Hypothyroidism

- About 85 % of the thyroid patients are hypothyroid
- Major cause of hypothyroidism in India is Iodine Deficiency Disorders (IDD)
- Salt iodization program is imposed by the Government of India to overcome the situation
- But if there any genetic problem of iodine utilization then iodized salt does not rescue the hypothyroidism

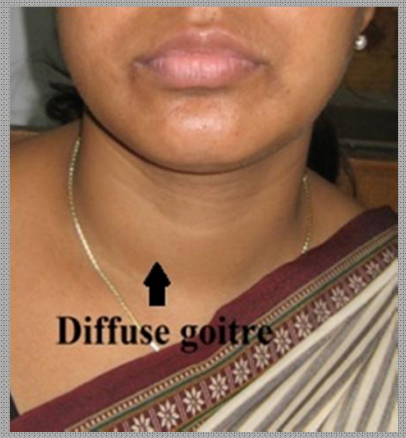
- About 15-20% of the hypothyroid patients harbor gene mutations
- Thyroid peroxidase (TPO) gene is important for iodine utilization as well as thyroid hormone production

Patient Data Sheet

General Complains	Adults	Thyroid swelling , Lethargy, Weight gain/Weight loss, Cold intolerance, Loss of Memory, Constipation
	Children	Delayed milestone like sitting without support , Speech , Walking
Exclusion Criteria		Iodine deficiency excluded by urine iodine estimation (10-20 µg/dL) Presence of Serum Anti-TPO antibody above the normal level (>60 IU/mL)

Clinical presentation in hypothyroid patients

Diffuse smooth goitre also called simple non toxic goiters. It refers to an enlargement of the thyroid that is NOT associated with inflammatory or neoplastic alterations



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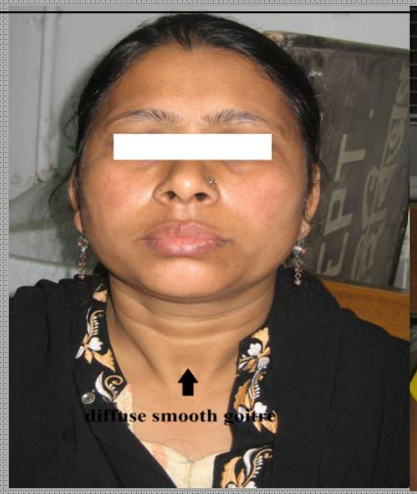

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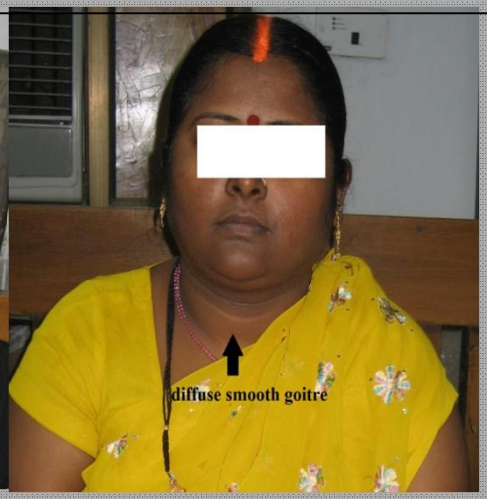

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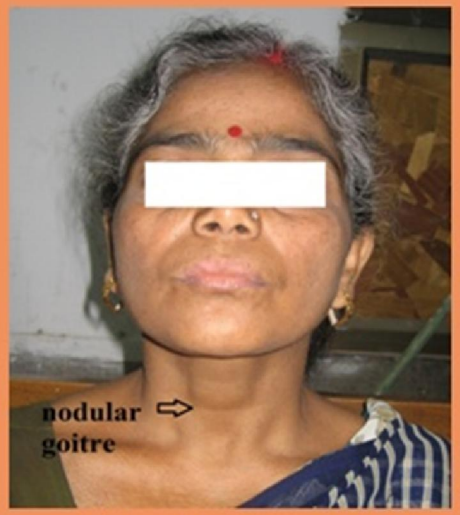

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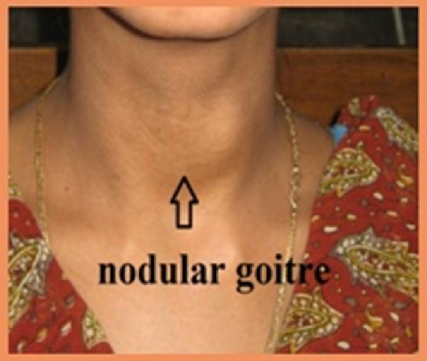

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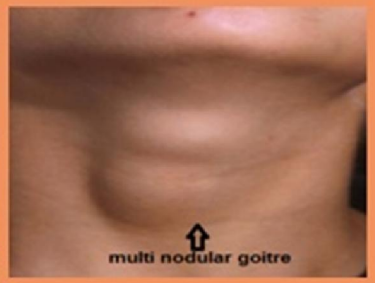


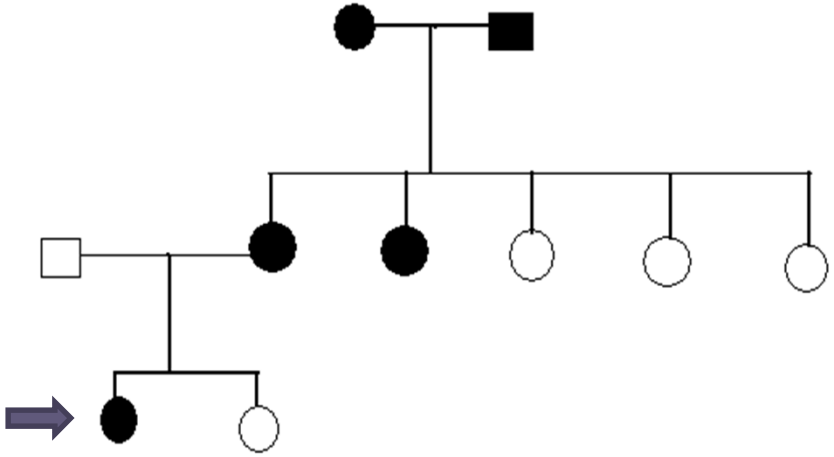
Patient id 147

Nodular goitre

Function of Thyroid Peroxidase (TPO)

- Iodide is trapped in thyroid gland, oxidized and bound to tyrosine to form iodotyrosines in thyroglobulin (TG) which generateT3 and T4
- TPO catalyzes the oxidation of iodide molecule and iodination of tyrosine residues in thyroglobulin.
- TPO is the rate limiting enzyme in the biosynthesis of thyroid hormones.
- Less production or activity of TPO causes hypothyroidism.

Representative PEDIGREE OF PATIENTS FAMILIES



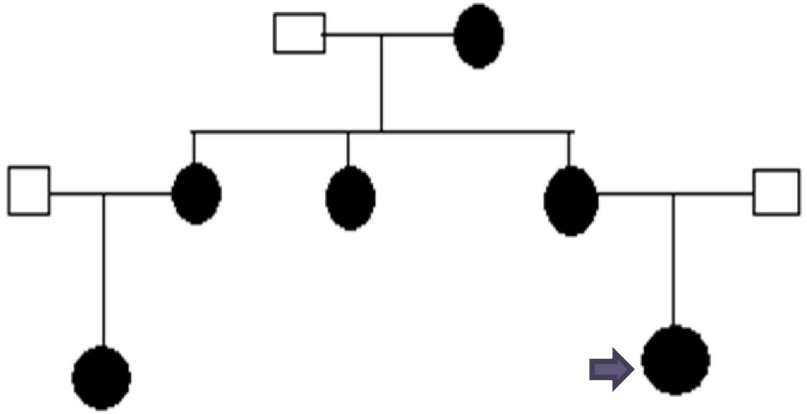
Female affected

Female Normal

Male affected

Male Normal

Patient Id: 46



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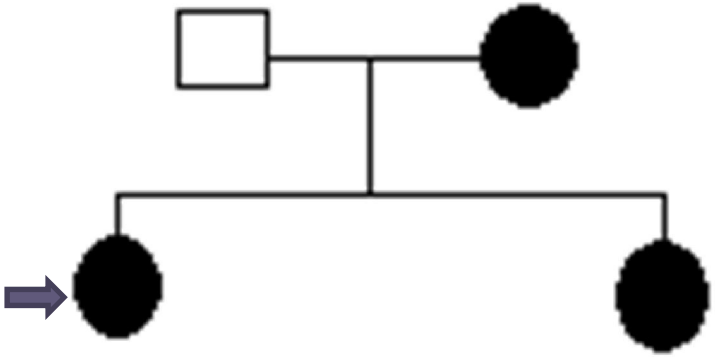


Patient Id: 67

Control

Age and Sex matched individuals with no goitre or clinical evidence of hypothyroidism

TSH level (<5.0 µU/ml)

Objectives

Estimation of association between genotype/allele frequencies at various polymorphic loci in TPO gene with clinical spectrum of thyroid dysfunction

SCREENING OF TPO GENE MUTATIONS

- Peripheral blood samples collected from the patient and normal individuals

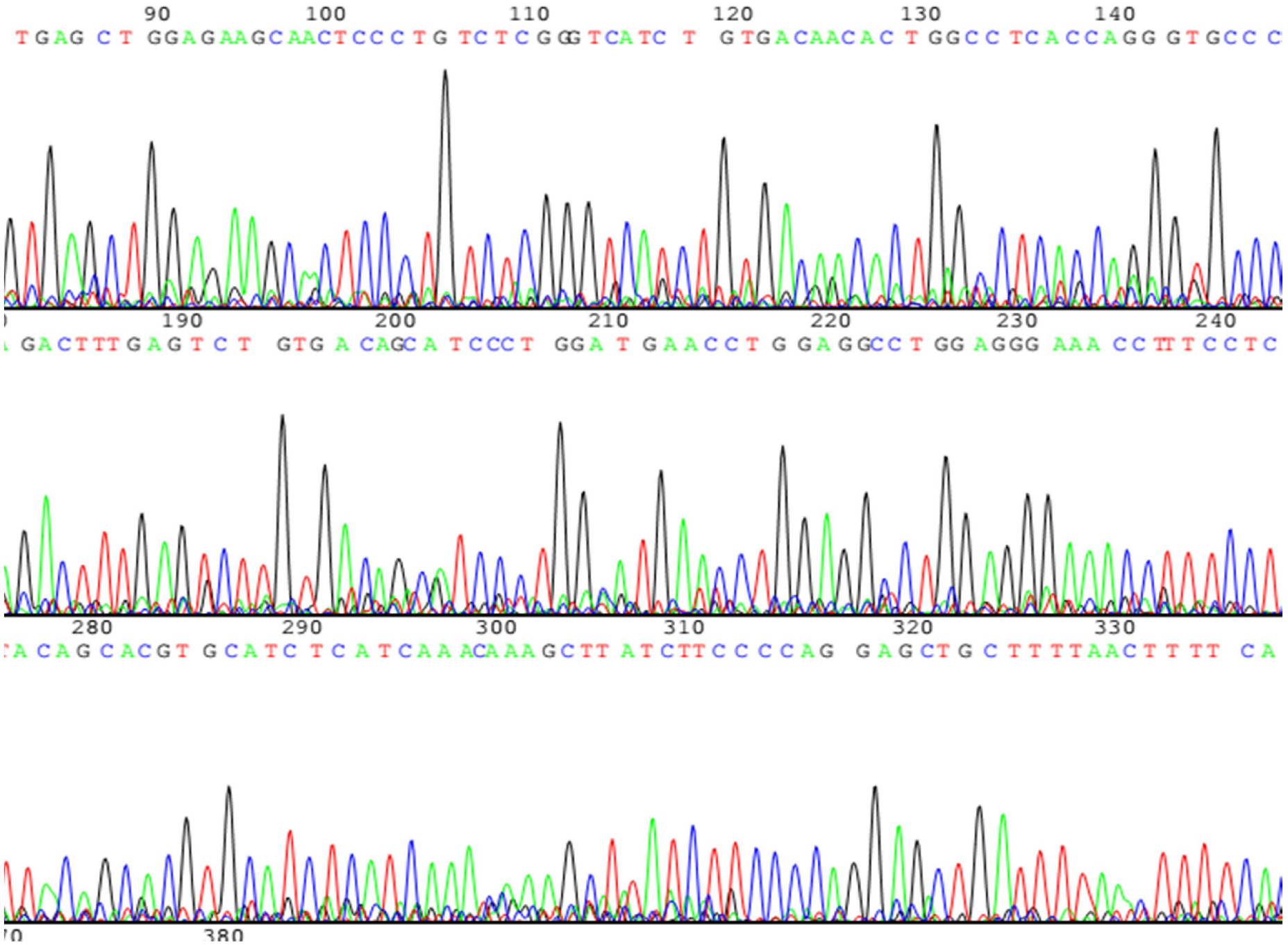
- Genomic DNA isolated from blood using QIAamp Blood Kit (QIAGEN, Hilden, Germany).

- PCR amplification were done for each of the selected Exons using specific primers and then Direct DNA sequencing.

- Sequences Alignment between sequences of case and control individuals was performed by using clustal W programme

we observed mutation in the exon 11 Thr 725 Pro (T725P). As we know the Thr is the phosphorylation site of the protein which important for the activation therefore, reduce the functional efficacy of the enzyme.

We are not clear about the effect of these intronic mutations in the function of TPO gene. But it may be hypothesized that the intronic mutations may have some regulatory role in the modulation of protein behavior.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190 200 210 220 230 240
GACTTTGAGTCT GTG A CAGCA TCCCT GGA T GAACCT G GAGGCCT GG AGGG AAA CCTTCCTC
280 290 300 310 320 330
CA CAG CACGT GCATC TCATCAAACAAAGCTT ATCTTCCCCCAG GAGCTG CTTTTAACTTTT CA
380

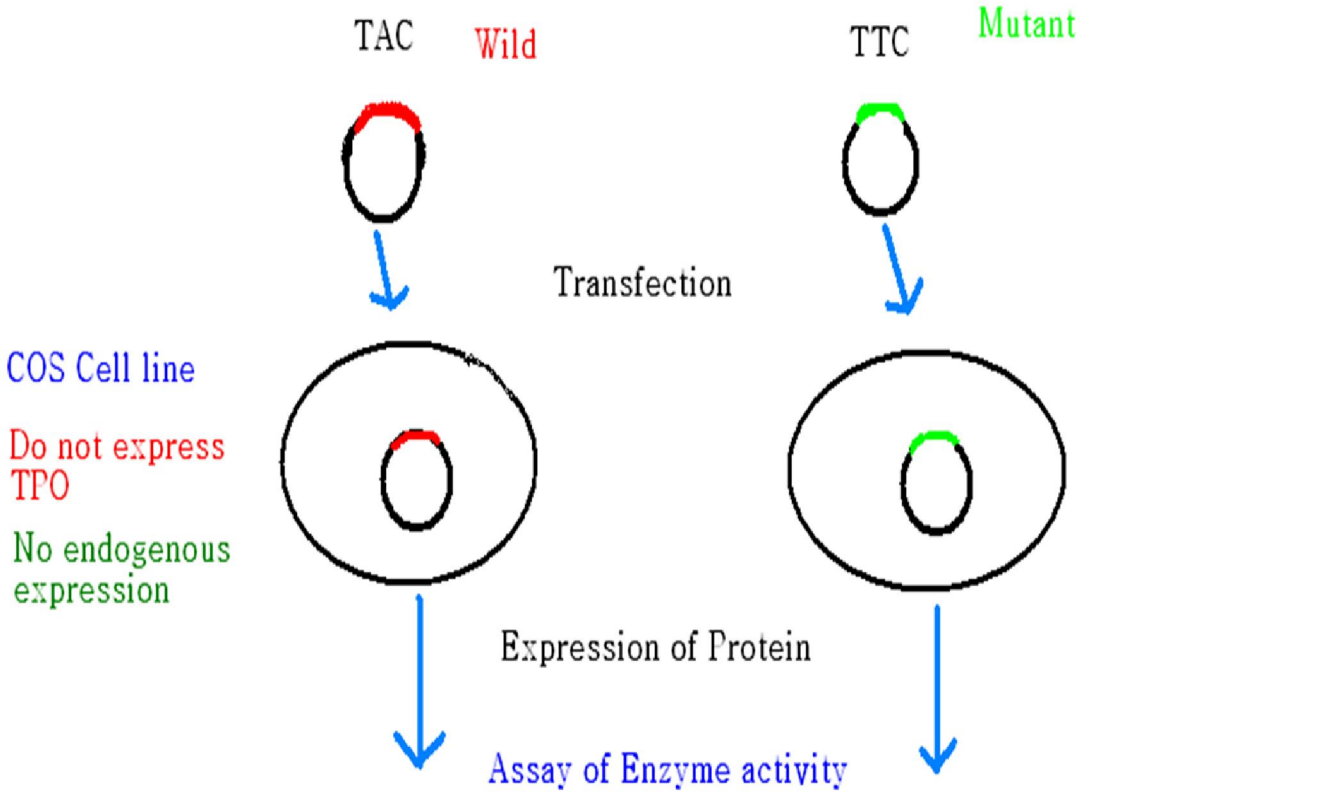
Functional *in vitro* analysis of TPO mutations

Polymorphisms in the TPO gene, identified in this study have been assayed in vitro. Essentially , a cDNA (supplied from Invitrogen) clone for TPO in a mammalian expression vector has been mutated by **site directed mutagenesis** to create clones representing patient mutations/polymorphisms.

We used pcDNA3.1 (vector) for TPO cDNA cloning. The recombinant plasmids (TPO cDNA + plasmid) were amplified in DH5 alpha competent cells.

Plasmid was purified by use of the plasmid isolation kit (PureLink™ Quick Plasmid Miniprep Kit, Invitrogen). We used Invitrogen Lipofectamine® Reagent for transfection in COS 7 cell line. COS 7cells were harvested with trypsin EDT A treatment and protein concentration was determined on a 100µl aliquot using the Bio-Rad protein assay (BioRad,Munchen, Germany).

Thirty micrograms of the deoxycholate extracted membrane protein fraction containing recombinant TPO and normal control TPO were electrophoresed on a 7.5% SDS-polyacrylamide gel. TPO protein was visualized using ECL Western blotting detection reagent (abcam®)



If Mutant type exhibit same enzyme activity as Wild type then the mutation is not related with the disease


Thank you ...