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Identification of Genetic Markers Using Polymerase Chain Reaction (PCR) in Graves' Hyperthyroidism

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1. Introduction

Thyroid is a butterfly shaped gland composed of two encapsulated lobes, located on either side of the trachea just below the cricoid cartilage. This is connected by thin isthmus and is composed of spherical thyroid follicles, which contain the hormone in colloidal form. T_3 and T_4 are active hormones secreted under the control of TSH from adenohypophysis of pituitary gland. T_3 is three to four fold more potent than T_4. It is involved in normal growth and development in children temperature regulation, metabolism, energy production and intelligence in both children adults. It ensures normal growth and development of nervous system [1].
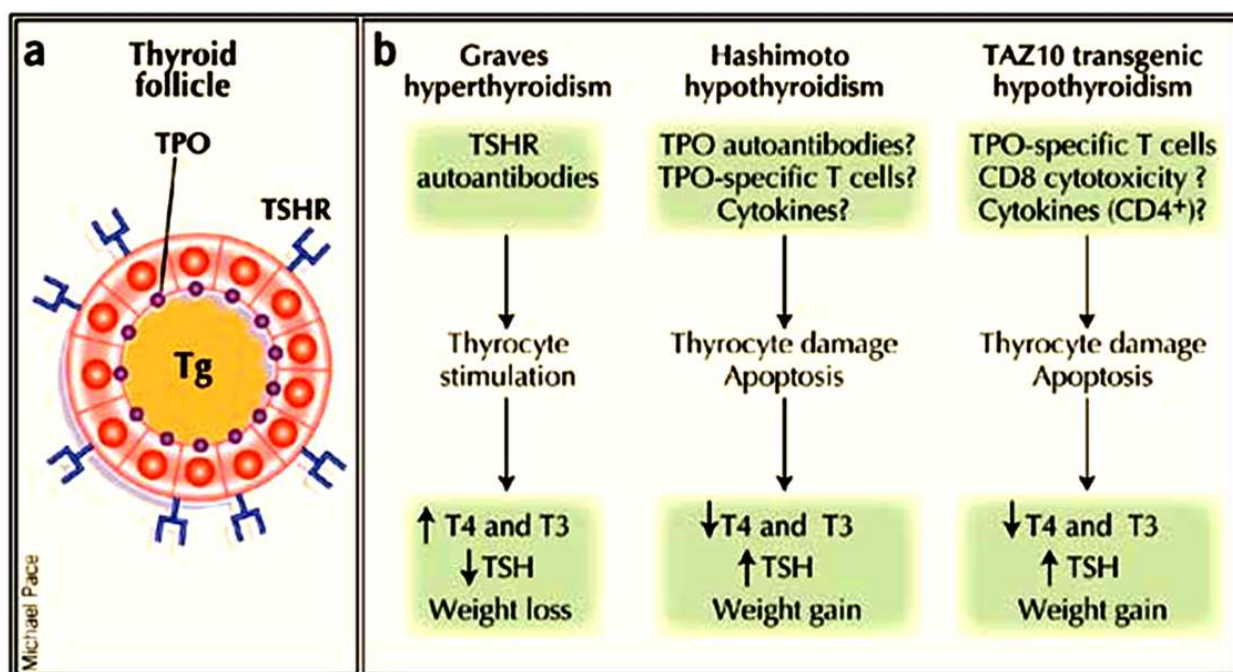

Fig. 1. Diagrammatic representation of variation of thyroid hormones in hypo and hyper thyroidism

The normal range of T_4 is suggested to be 77-155nmol/L, T3 to 1.2 -2.8nmol/L) and TSH to be 0.3-4 mU/L [2]. If the hormone levels are above or below the normal range, it leads to hyperthyroidism or hypothyroidism. The most common hypothyroid condition is Hashimoto's thyroiditis in adults and congenital hypothyroidism in children. Hyperthyroid conditions include Graves' disease, postpartum thyroiditis and thyrotoxicosis factitia.

Hyperthyroidism also leads to a number of complications like heart problems, brittle bones (Osteoporosis), eye problems (Graves' opthalmopathy) (Figure:2).
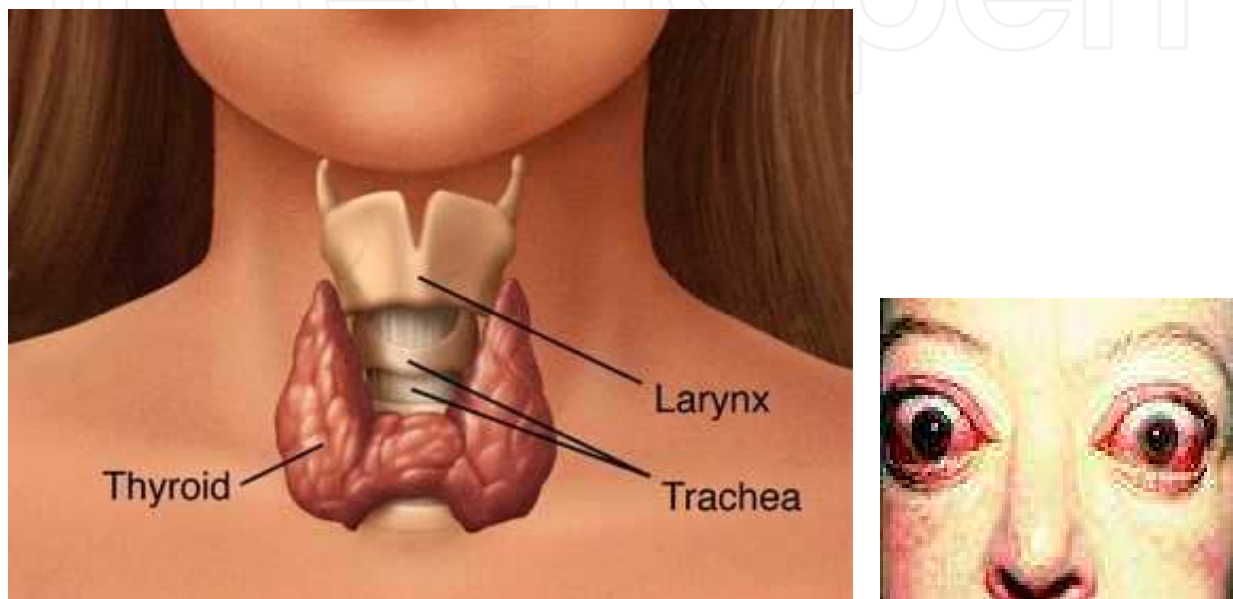

Fig. 2. Symptoms of Graves' disease

Hypothyroidism describes an under active thyroid gland that is producing low level of thyroid hormone. Hypothyroid patients experience a variety of symptoms, including weight gain, intolerance to cold, goiter (enlarged thyroid), dry coarse, skin, fatigue, constipation, decreased heart rate, poor memory and depression.

The most common form of hyperthyroidism is Graves' disease (GD), an autoimmune disorder accounting for 60-80 % of all cases, in which the antibodies produced by immune system stimulates thyroid gland to produce excess of thyroxine. Normally, the immune system uses antibodies to protect against viruses, bacteria and other foreign substances that enter the body system. In GD, the antibodies mistakenly attack the thyroid gland and occasionally the tissues behind the eyes and the skin of lower legs over the shins. Though the exact cause of GD is not known, several factors including a genetic predisposition are likely to be involved **(Figure:3).**

GD is an organ specific heterogeneous autoimmune disorder associated with T-lymphocyte abnormality affecting the thyroid eyes and skin. GD is also multifactorial disease that develops as a result of complex interaction between genetic susceptibility genes and environmental factors. Human leucocyte antigen (HLA) and cytotoxic T-lymphocyte associated molecule-4 (CTLA-4) are susceptibility candidates. CTLA_4 gene plays an important role in the development of GD, which is located on chromosome 2 q33.
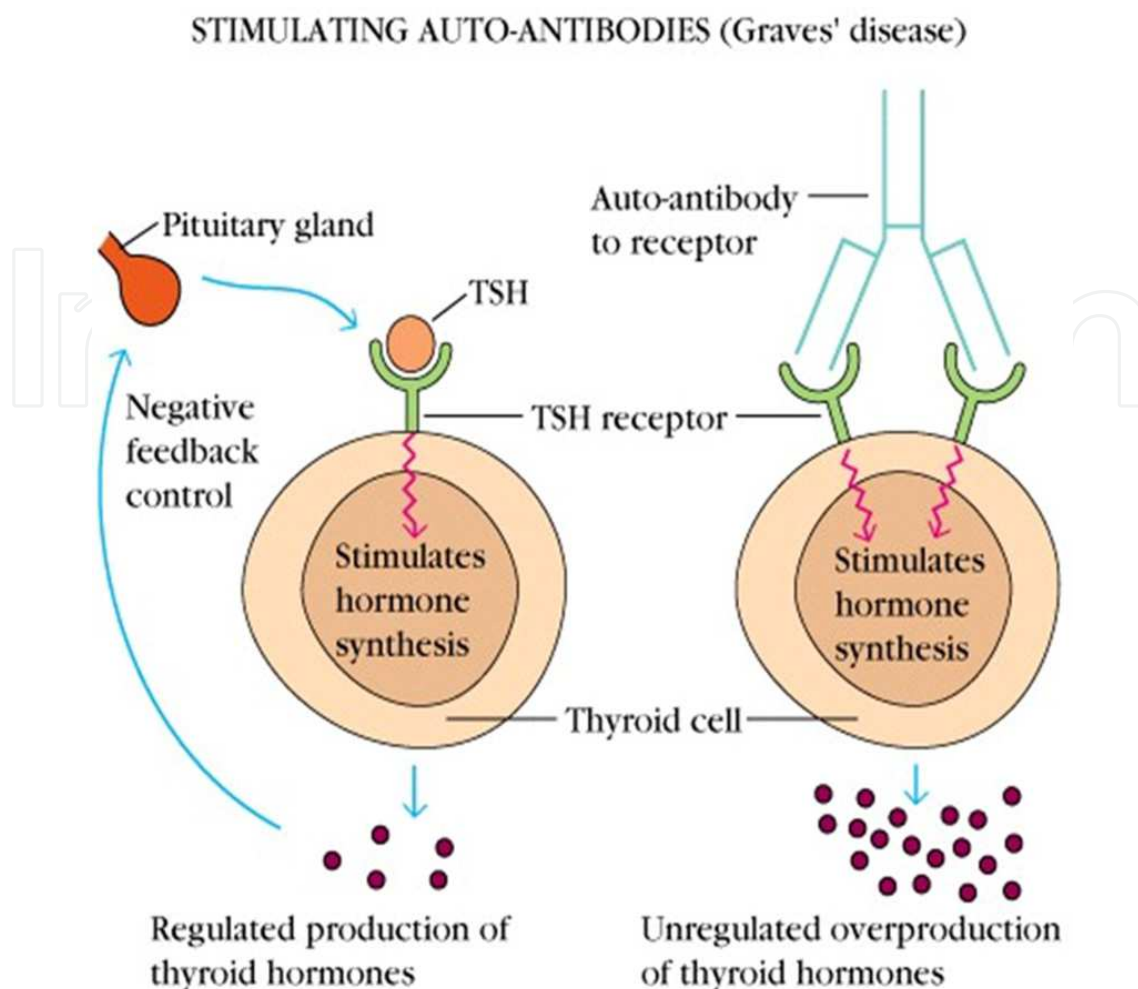
STIMULATING AUTO-ANTIBODIES (Graves' disease)

Fig. 3.

2. Cytogenetic location of CTLA-4 gene

Cytogenetic Location: 2q33

Molecular Location on chromosome 2: base pairs 204,732,510 to 204,738,682
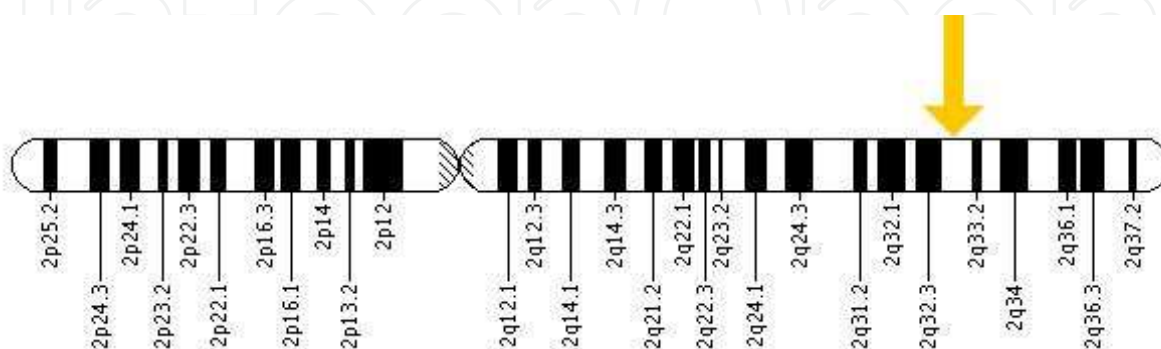

Fig. 4. The CTLA4 gene is located on the long (q) arm of chromosome 2 at position 33.

More precisely, the *CTLA4* gene is located from base pair 204,732,510 to base pair 204,738,682 on chromosome 2.

Activation of T cells requires 2 signals transduced by the antigen specific TCR and co stimulatory ligand such as CD28. CTLA-4, which is expressed on activated T cells, bind to B7 present on antigen presenting cells and functions as a negative regulator of T cell activation. CTLA-4 gene polymorphism confers susceptibility to several autoimmune diseases, such as Graves' disease (GD), Hashimoto's thyroiditis (HT), Addison's disease (AD), Insulin-dependent diabetes mellitus (IDDM), Rheumatoid arthritis (RA) and Multiple sclerosis.

The activity of T cells requires a co stimulatory signal mediated by CD28/B7 interaction. The CTLA-4 gene product delivers a negative signal to T cells and mediates apoptosis. This CTLA-4 gene product is a T cell surface molecule that binds to the B 7 molecule on the antigen presenting cells (APCs). The CTLA-4 gene expression on T cells may affect the course of ongoing immune process. TSH receptor antibody (TRAb) causes Graves' hyperthyroidism.

The GD will go into remission during antithyroid drug (ATD) treatment. Remission of GD is predicted by a smooth decrease in TRAb during (ATD) treatment. Treatment of GD may involve surgery or use of radioactive iodine or use of ATD like propylthiouracil, methimazole and carbimazole. The genetic susceptibility to GD is also conferred by genes in human leucocyte antigen (HLA) and several other genes that are not linked to HLA. The present paper describes the association of GD with the CTLA-4 gene.

The prevalence of hyperthyroidism has been reported to be 3.63% and hypothyroidism to be 2.97% especially the females being more affected by hyperthyroidism [3]. Hence the current study deals with A/G single nucleotide polymorphism (SNP) at position 49 (exon1, codon 17) of the CTLA-4 gene where in Thr/Ala substitution and can be a function related marker. It has been shown to be associated with GD in Caucasians, Japanese, Koreans, Tunisians, Hong Kong Chinese children [2,4,5,6,7,8,9,10] and South Indains [11,12].

The polymorphism cited (A/G polymorphism in exon 1, C/T polymorphism in the promoter, and micro satellite repeat in 3'-untranslated region of exon 4) in CTLA-4 gene have been reported to be associated with autoimmune endocrine disorder.

A/G polymorphism at position 49 in exon 1 of the CTLA-4 gene among South Indian population with Graves' hyperthyroidism has revealed the frequencies of the GG genotype and "G" allele to the significantly higher in GD patients. The study has also demonstrated that GD patients had higher frequencies of "G" allele (GG genotype) and lower frequencies of "A" allele (AA genotype) than control group.

Kinjo *et al.,* (2000) have also reported the relationship between the CTLA-4 gene type and severity of the thyroid dysfunction. At diagnosis, free T4 concentrations were shown to be more in patients with the GG genotype and low in patients with the AA genotype. GD patients were reported to have more "G" allele than the control, suggesting that the CTLA-4 GG genotype might induce down regulation of T-cell activation. If the function of CTLA-4 with "G" alleles at position 49 in exon 1 is impaired CTLA-4 function may have d\difficulty in achieving remission.

Identification of SNP

We can analyze and identify all types of gene SNPs by Polymerase Chain Reaction (PCR) thermal cycler.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a laboratory (in vitro) technique for generating large quantities of a specified DNA. Obviously, PCR is a cell-free amplification technique for synthesizing multiple identical copies (billions) of any DNA of interest, which was developed in 1994 by Karry Mullis (Nobel Prize, 1993). PCR is now considered as a basic tool for any molecular biologist.

3. Primer designing [13]

As oligonucleotide primers are useful for polymerase chain reaction (PCR), oligo hybridization and DNA sequencing, proper primer designing is actually one of the most important factors/steps. Various bioinformatics programs are available for selection of primer pairs from a template sequence.

3.1 Guidelines for primer design

When choosing two PCR amplification primers, the following guidelines should be considered:

Primer length: It is accepted that optimal length of PCR primers is 18-22 bp (Wu et al., 1991)

Melting temperature (Tm): It can be calculated using the formula of Wallace et al., 1997, Tm (^{o}C) = 2(A+T)+4(G+C). The optimal melting temperature for primers ranges between 52-58°C. Primers with melting temperature above 65°C should also be avoided because of potential for secondary annealing.

Primer annealing temperature: The two primers of a primer pair should have closely matched melting temperatures for maximizing PCR product yield. The difference of 5°C or more each can lead to no amplification.

$$T_a = 0.3 \times T_m \left(primer \right) + 0.7 \qquad \left(T_m primer = 14.9 \right)$$

Where, Tm (primer) = Melting Temperature of the primers, T_m (product) = Melting temperature of the product.

GC Content: Primers should have GC content between 45 and 60 percent. GC content, melting temperature and annealing temperature are strictly dependent on one another

.Dimers and false priming because misleading results: Presence of the secondary structures such as hairpins, self dimer produced by intermolecular or intramolecular interactions in primers can lead to poor or no yield of the product.

Avoid Cross homology: To improve specificity of the primers it is necessary to avoid regions of homology

3.2 Software for primer design

NETPRIMER is software used to design and analyze the parameters of designed primer sequences using the following link http://premierbiosoft.com/netprimer/index.html

3.3 PCR standardization [13,14]

PCR is a revolutionary technique used in almost all molecular biology experiments. In PCR, the repeated three-step process of denaturation, primer annealing and DNA polymerase extension results in exponential amplification of target DNA. Initially PCR was reported with E.Coli DNA polymerase Klenow fragment in 1985. In 1988, the first report on PCR using thermostable Taq DNA polymerase was published. Since then PCR has been extensively modified and used for various applications such as cloning, sequencing, site-directed mutagenesis, diagnostics, genotyping, genome walking, amplification of RNA after reverse transcription for gene expression analysis amplification of a whole genome, etc.

The central components of a PCR reaction are oligonucleotide primers, thermostable DNA polymerase, target DNA, dNTPs and reaction buffer including $MgCl_2$. When a new PCR has to be developed, suitable primer pairs should be designed based on the target sequence,. Subsequently, the concentration of PCR components and the cycling conditions should be optimized.

Thermostable enzymes:

Thermostable enzymes should be selected based on the applications. High fidelity Taq DNA polymerase and proofreading recombinant enzymes are required for the amplification of more than 3 kb target sequence. For a standard PCR, 2 to 5 units of Taq DNA polymerase are recommended for a typical 100µl PCR.

Deoxynucleoside triphosphate (dNTPs):

For a standard PCR, 100 to 200 µM concentrations of dNTPs is used. The balanced solutions of all four nucleotides should be used to minimize the error frequency. The concentrations may be increased for Multiplex PCR and Repetitive PCR, where more than one PCR amplicons are expected.

Template DNA:

The purity and concentration of the template DNA are critical for a successful PCR amplification. For initial experiments, 0.1 to 200ng of the template DNA, based on the type can be used. For example, if it is a plasmid 0.1 to 1ng is sufficient. If the template is human genomic DNA, upto 200ng can be used.

Primer concentrations:

The primer concentration can affect the PCR. If the primer concentration is too low, amplifications will be failed; and if the concentration is too high, non-specific amplification will occur. Therfore, the primer concentration should be optimized empirically between 0.1 to 1µM final concentrations. The most straightforward way of optimizing a PCR with a given primer pair is to change the concentration of $MgCl_2$ or the annealing temperature or both.

Optimization of primer annealing temperature:

Optimization of the primer annealing temperature is the most critical step in PCR. The primer designing programs will suggest the Tm of the primers. In general, the annealing temperature should be set 2 to 5°C below the Tm of the primers. However, some oligonucleotides may not work optically at this temperature and hence the annealing temperature should be optimized using gradient PCR approach.

Optimization of MgCl₂ concentration:

Magnesium chloride is an essential component for PCR. It is a cofactor for Taq DNA polymerase. Mg^{++} promotes DNA/DNA interactions and forms complexes with dNTPs that are the actual substrates for Taq polymerase. When Mg++ is too low, primers fail to anneal to the target DNA. When Mg^{++} is too high, the base pairing becomes too strong and the amplicon fails to denature completely when you heat 94ºC. MgCl₂ concentration should be optimized for every PCR reaction. All the components of the reaction mixture can bind to magnesium ion, including primers, template, PCR products and dNTPs. Therefore, the concentration of MgCl₂ has to be optimized for a new PCR. The most commonly used concentration of MgCl₂ is 1.5mM and it can be optimized empirically between 1.5 and 4.0mM.
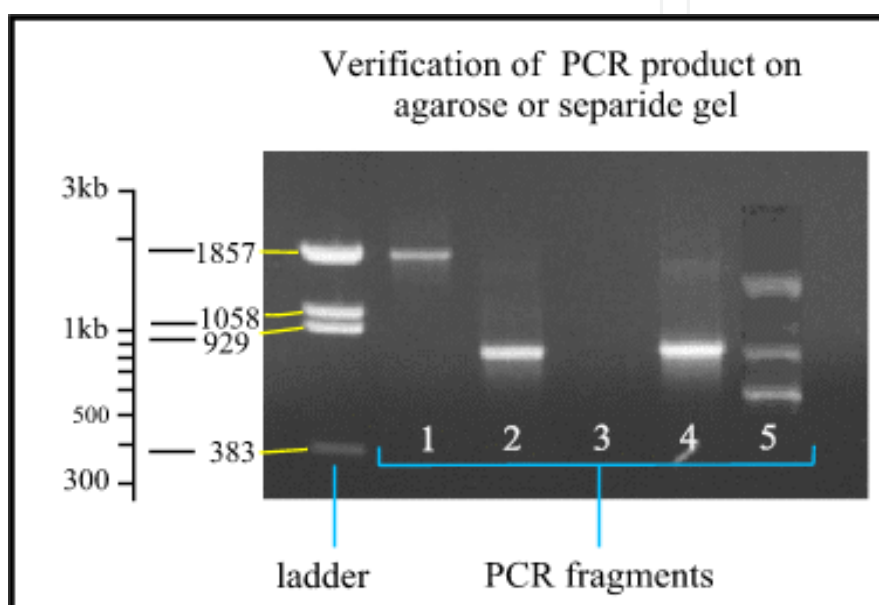

Fig. 5.

3.4 Genetic marker [15,16,17,18]

A **genetic marker** is a gene or DNA sequence with a known location on a chromosome that can be used to identify cells, individuals or species. It can be described and observed as a variation which may arise due to mutation or alteration in the genomic loci. A genetic marker may be a short DNA sequence, such as a sequence surrounding a single base-pair change (single nucleotide polymorphism, SNP), or a long one, like minisatellites. For many years, gene mapping was limited in most organisms by traditional genetic markers which include genes that encode easily observable characteristics such as blood types or seed shapes.

3.5 Some commonly used types of genetic markers

RFLP (or Restriction fragment length polymorphism)
SSLP (or Simple sequence length polymorphism)
AFLP (or Amplified fragment length polymorphism)
RAPD (or Random amplification of polymorphic DNA)
VNTR (or Variable number tandem repeat)

Microsatellite polymorphism, SSR (or Simple sequence repeat)
SNP (or Single nucleotide polymorphism)
STR (or Short tandem repeat)
SFP (or Single feature polymorphism)
DArT (or Diversity Arrays Technology)
RAD markers (or Restriction site associated DNA markers)
They can be further categorized as dominant or co-dominant.

Dominant markers allow for analyzing many loci at one time, e.g. RAPD. A primer amplifying a dominant marker could amplify at many loci in one sample of DNA with one PCR reaction. The dominant markers, as RAPDs and high-efficiency markers (like AFLPs and SMPLs), allow the analysis of many loci per experiment within requiring previous information about their sequence.

Co-dominant markers analyze one locus at a time. A primer amplifying a co-dominant marker would yield one targeted product. so they are more informative because the allelic variations of that locus can be distinguished. As a consequence, we can identify linkage groups between different genetic maps but, for their development it is necessary to know the sequence (which is still expensive and is considered one of their down sides). **Eg.** RFLPs, microsatellites, etc.,

3.6 Uses of genetic markers

- Genetic markers can be used to study the relationship between an inherited disease and its genetic cause (for example, a particular mutation of a gene that results in a defective protein). It is known that pieces of DNA that lie near each other on a chromosome tend to be inherited together. This property enables the use of a marker, which can then be used to determine the precise inheritance pattern of the gene that has not yet been exactly localized.
- Genetic markers have to be easily identifiable, associated with a specific locus and highly polymorphic, because homozygotes do not provide any information.
- Detection of the marker can be direct by RNA sequencing, or indirect using allozymes.
- Genetic Markers have also been used to measure the genomic response to selection in livestock.
- Natural and artificial selection leads to a change in the genetic makeup of the cell. The presence of different alleles due to a distorted segregation at the genetic markers is indicative of the difference between selected and non-selected livestock.

Hence, SNP (Single nucleotide polymorphism) in Graves' hyperthyroidism is used as marker to identify which mutation is responsible for causing GD and other hereditary diseases.

4. Analysis of CTLA-4 A/G polymorphism among South Indian population

4.1 Protocol used for A/G single nucleotide polymorphism (SNP) study in Graves' disease

Genomic DNA was prepared from peripheral white cells using standardized protocol. We have analysed CTLA -4 genotypes and allele with PCR. PCR was performed with

oligonucleotide primers (Forward, 5' – GCTCTACTTCCTGAAGACCT – 3' and Revers, 5' – AGTCTCACTCACCTTTGCAG – 5')[2]. PCR was performed by initial denaturation 30 sec for 5 min. annealing for 45 sec at 57°C, extension for 30 sec at 72°C, denaturation 30 sec at 94°C (for 20 cycles) and final extension for 7 min at 72°C. The PCR product was confirmed by agarose (1.8%) gel electrophoresis. The presence of G alleles was determined in each subject by PCR amplification of CTLA-4, followed by diffusion with *Bbv1,* which acts on the G variation, but not on the A variation. It a G allele was at position 49, 88/74 bp fragments were obtained. This was confirmed by 2% agarose gel.

4.2 Restriction digestion

The amplified CTLA-4 gene should be digested with the restriction enzyme *Bbv1,*which is commercially available. A typical 30µl reaction mix was used . Modify the required volume proportionately.

PCR amplified product	– 20. 0µl
10x buffer	- 3.0µl
Bbv1(10units/ul)	- 1.0µl
Deionized water	- 6.0µl
Total	- 30.0µl

Incubated the reaction mixture at 37°C for 4 hrs and inactivated by heating at 70°C for 10 min. The product was confirmed using 2% agarose gel electrophoresis.

4.3 Results

The presence of genomic DNA confirmed by subjecting the agarose gel electrophoresis (0.7%) **(Figure 6).** The genomic DNA was then subjected to PCR and 162 bp fragments were obtained **(Figure 7).** The amplified PCR product digested with enzyme ***Bbv*1,** the restriction enzyme acts on the G variation, but not on the A variation. If a G allele was at position 49, 88bp and 74bp fragments were obtained and the fragments were detected by 2% agarose gel electrophoresis **(Figure 8).**

In the present study, the G/G genotype was observed in 32 (40 %) GD patients and in 26 (32.50 %) individuals of the control group, A/G genotype was found in 37 (46.25 %) patients and in 25 (31.25 %) persons of the control group, A/A genotype was observed in 11 (13.75 %) patients and in 29 (36.25 %) persons of the control group and G allele was found in 50 (62.5%) GD patients and in 38 (47.5 %) persons of the control group, and A allele was found in 30 (37.5 %) GD patients and 42 persons (52.5%) of the control group **(Table 1)**. There was significant difference (p <0.05) in genotype and allelic frequency between the control group and GD patients. The present study also demonstrates an association between the CTLA-4 gene polymorphism in Graves' disease and with the remission rate of Graves' hyperthyroidism. Among the GD cases studied, only 2% had remission. The frequencies of GG genotype (40 %) and G allele (62.5%) were higher when compared to A/A genotype (13.75%) and A allele (37.5 %) (Table 1).
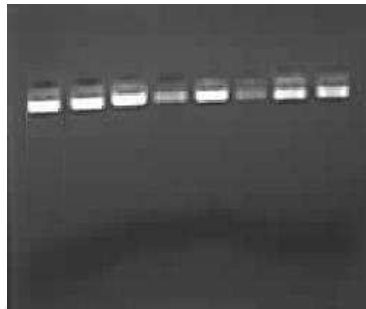
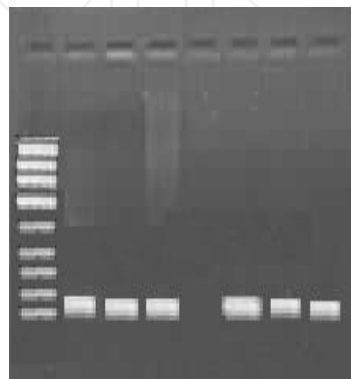Fig. 6. Confirmation of human genomic DNA

Fig. 7. CTLA-4 gene amplification

Fig. 8. Restriction analysis of CTLA-4 gene

GENOTYPE	GD patient (n=80)	*Control group* *(n=80)*
G/G	32 (40%)	26 (32.50%)
A/G	37 (46.25%)	25 (31.25%)
A/A	11 (13.75%)	29 (36.25%)
Allele		
G	50 (62.5%)	38 (47.5%)
A	30 (37.5%)	42 (52.5%)

Table 2. Prevalence of CTLA-4 gene genotype and allele frequency among South Indian

4.4 Discussion

In the present study genomic DNA was isolated from patients and control groups and was subjected to Agarose gel electrophoresis (0.7%). This enables easy visualization of DNA band patterns. After confirming the presence of genomic DNA, it was subjected to PCR and 162 bp fragments were obtained. The amplified PCR product was digested with enzyme *Bbv*1. The restriction enzyme acts on the G variation, but not on the A variation. If a G allele was at position 49, 88bp and 74bp two fragments were obtained. The PCR products were detected by 2% Agarose gel electrophoresis.

A/G polymorphism at position 49 in exon 1 of the CTLA-4 gene among Madurai population with Graves' hyperthyroidism revealed that the frequencies of the GG genotype and G allele were significantly higher in GD patients. This study has also revealed lower frequency (or absence) of A allele (AA genotype) than the control. CTLA-4 gene polymorphism has been reported to be associated with GD. CTLA-4 molecule is a member of the family of cell surface molecule CD28, which binds to B7. The CTLA-4/B7 complex competes with the CD28/B7 complex and delivers negative signals to the T-cells, which affects T-cell expansion, cytokine production, and immune responses as evidenced by Park *et.al.*[6] in Korean population, Yanagawa *et al.* [9] in Japanese population and Yanagawa *et al*. [8] in Caucasian population. However, we do not know how CTLA-4 gene polymorphisms may contribute to the development of Graves' hyperthyroidism.

Three polymorphism sites (A/G polymorphism in exon 1; C/T polymorphism in the promoter, and micro satellite repeat in the 3'-untranslated region of exon 4) in the CTLA-4 gene have been reported to be associated with autoimmune endocrine disorders. Kinjo *et. al.*, [2] have reported the relationship between the CTLA-4 gene type and severity of the thyroid dysfunction. At diagnosis, free T_4 concentrations were shown to be highest in patients with the GG genotype and lowest in patients with the AA genotype. GD patients have more G allele than control, suggesting that the CTLA-4 GG genotype might induce down regulation of T-cell activation. If the function of CTLA-4 with the G alleles at position 49 in exon 1 was impaired CTLA-4 function might have difficulty in achieving remission.

	Graves' Disease % (n = 144)	Controls % (n = 110)
Genotype		
G/G	50 (34.7)	26 (23.6)
A/G	62 (43.1)	46 (41.8)
A/A	32 (22.2)	38 (43.6)

Table 3. Frequency of the genotype and allele of A/G polymorphism at position 49 in exon 1 of CTLA-4 gene in GD patients and controls among Japanese –population. [2]

Bednarczuk *et. al.*, [4] analysed the association of CTLA-4 A49G polymorphism with Graves' disease in Caucasian and Japanese population. Their study also reveals that, CTLA –4 G allele and G/G genotype confer genetic susceptibility to GD in Caucasian and Japanese population.

The study of Kouki *et. al.*, [5] among patients with GD revealed there were more individuals with G/G (17.8 %GD vs 11.6% of controls) or A/G CTLA-4 exon 1 genotypes (64.4 % GD vs 53.5% control) and significantly fewer individuals with the A/A alleles (17.8 %GD vs 43.9

%control) when compared with controls. According to their findings, the frequency of the G allele was higher in GD patients (50%) than in controls (38.4%) in their population.

There was significant difference between the control group and GD patients both in genotype and allelic frequency. Therefore, in accordance with previously published results, the present study also demonstrates an association between the CTLA-4 gene polymorphism in Graves' disease and with the remission rate of Graves' hyperthyroidism. Among the GD cases studied, only 2% had remission and the frequencies of GG genotype and G allele were higher when compared to A/A genotype and A allele. GD patient with G allele in exon 1 of the CTLA-4 gene were required to continue Anti thyroid drug (ATD) treatment [19] for longer periods to achieve remission. Further studies will be required to determine a clear association of the CTLA-4 gene polymorphism with the remission of GD.

We have studied another gene polymorphism called PKD1 (C/T) at position 4058 in exon 45 which is responsible for causing autosomal polycystic kidney disease (ADPKD) among South Indian.

5. Short summary of C/T polymorphism in PKD1 gene

Polycystic kidney disease (PKD) is a group of monogenic disorders that result in renal cyst development in kidney leads to kidney failure. Autosomal dominant polycystic kidney disease (ADPKD) and autosomal recessive polycystic kidney disease (ARPKD) are two forms of PKD, which are largely limited to the kidney and liver, which extends from neonates to old age. ADPKD is a commonly inherited disorder in humans, with a frequency among the general population of 1 in 500. ADPKD caused by mutations in PKD1 gene (85%) located on human chromosome 16p13.3; the remaining 15% are caused by mutations in the PKD2 gene, located on human chromosome 4q21-23. A total of 60 ADPKD patients among South Indian (Madurai) population were analyzed. In genetic study, the genomic DNA was isolated, which would be subjects into PCR (Figure:9) and RFLP analysis (Figure:10). C/T polymorphism at position 4058 in exon 45 of the PKD1 gene among South Indian (Madurai) population with ADPKD revealed that the "TT" "CT" genotype and the frequency of "T" allele was found be significantly (at p=0.001) higher in the patients compared to control subjects. The study was demonstrated that ADPKD patients had higher frequencies of "T" allele and lower frequency of "C" allele than control subjects. The present study also has been supported by Constantinides et al.,[20]. Therefore, the study reveals that there was an association of C/T polymorphism in ADPKD and the prevalence of ADPKD among South Indian (Madurai) population.
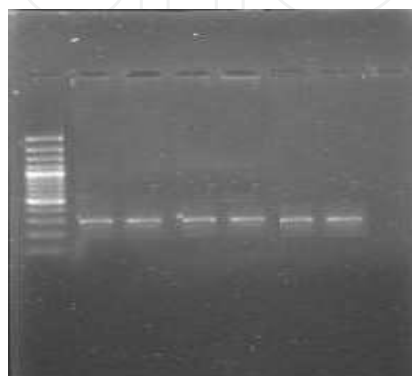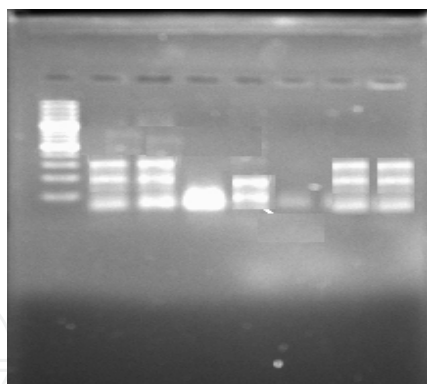

Fig. 9. PKD1 gene amplification

Fig. 10. Restriction digestion PKD1 gene

Hope this chapter will provide an insight on genetic screening of different disease and genetic disorders.

6. Acknowledgement

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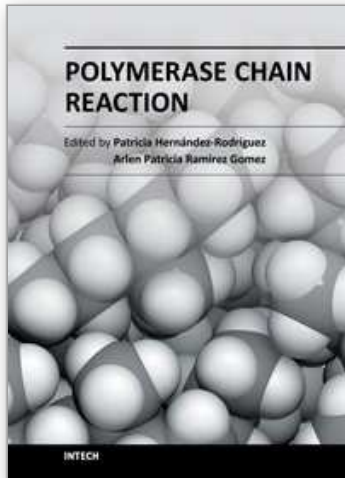
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