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Genetic association of *TSHR* gene polymorphism with Autoimmune Thyroid Disease (AITD) in Vindhyan region of Madhya Pradesh

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Abstract: The thyroid stimulating hormone receptor (TSHR) represents the primary autoantigen in GD, in which autoantibodies bind to the receptor and mimic its ligand, thyroid stimulating hormone, causing the characteristic clinical phenotype. Although early studies investigating the TSHR and GD proved inconclusive, more recently we provided convincing evidence for association of the TSHR region with disease. Intronic thyroid-stimulating hormone receptor polymorphisms have been associated with the risk for both Graves' disease and Graves' ophthalmopathy, but results have been inconsistent among different populations. We aimed to investigate the influence of thyroid-stimulating hormone receptor intronic polymorphisms in a large well characterized population of GD patients.

The Autoimmune Thyroid Disease (AITD) patients had markedly higher levels of FT3 (P=0.0192*) and ratio of FT3/FT4 (P<0.0001*) whereas low level TSH (P<0.0001***) were significantly associated to Autoimmune Thyroid Disease (AITD). Whenever, rests of all parameters were not significantly different between patient and healthy population. Genotype frequency distribution between case and control group was significantly associated with Autoimmune Thyroid Disease (χ^2 = 0.0174*, P= 8.103). In addition, Allele frequency (χ^2 = 3.287, P= 0.0698) and carriage rate (χ^2 = 2.269, P=0.1320) between both groups was not significantly associated to Autoimmune Thyroid Disease. allele 'A' was found little lower frequency in disease group as compared to HC group whereas allele '*G'* was present in little high frequency in the disease group but the difference is nominal and was not significant (χ^2 = 3.287, P= 0.0698). Carriage rate of allele 'G' was slightly high in Autoimmune Thyroid Disease.**

Keywords: BMI, TSHR gene, GD, FT3/FT4, AITD.

INTRODUCTION

The thyroid stimulating hormone receptor (TSHR) is primarily expressed on the thyroid follicular cell surface membrane and via its ligand, TSH, is one of the key regulators of thyroid growth and hormone production. TSHR autoantibodies with either stimulator (TSAb) or blocking (TBAb) activity are a key feature of autoimmune thyroid disease (AITD), with TSAb having a predominant effect in Graves' disease (GD) leading to hyperthyroidism. Genetic variation within the TSHR region may influence TSHR structure, expression and/or post-translational processing, which in turn could initiate or exacerbate the autoimmune response against the TSHR in GD [1, 3]. Original TSHR genetic studies focused on several nonsynonymous single nucleotide polymorphisms (nsSNPs) and despite a number of studies, no replicable GD associations emerged. Genome wide linkage analysis subsequently suggested a GD locus in this chromosome region. Graves' disease (GD), the most important of all clinical hyperthyroidism causes, is a common autoimmune thyroid disease affecting 0.5–1.0 % of the general population. GD results from the presence of autoantibodies to the thyroid-stimulating hormone receptor (TSHR), leading to over-activity of the thyroid gland. About 25–50 % of GD patients have clinical signs of Graves' ophthalmopathy (GO), most having mild disease. Genome-wide screens conducted in GD have identified a number of putative susceptibility genes in GD. About 20 identified genetic polymorphisms, including both thyroid-specific genes and those regulating the autoimmune response were already investigated. However, the analyzed variants showed little impact on the development of GD. For the present, the role of genetic factors in development of GO so far remains unknown. The TSHR has long been considered an important disease- specific susceptibility gene for both GD and GO **[2-5].**

TSHR glycoprotein is expressed on the cell membrane of thyroid follicles and is responsible for the brisk

immune activation of preadipocytes/adipocytes in the orbital space, which causes cell proliferation and an increased deposition of extracellular matrix leading to the expansion of orbital tissue. Genetic variation within the TSHR region may influence the protein structure and the expression and/or post-translational processing, which in turn may initiate or exacerbate the autoimmune response against the TSHR in GD **[4].** The influence of polymorphisms of the *TSHR* gene in the susceptibility to GD differs in distinct ethnic groups and conflicting results has been described, especially in relation to GO. Previous studies recently demonstrated that intronic polymorphisms have gained attention, especially because of their possible roles in the regulation of TSHR expression. Intronic polymorphisms are responsible for generation of different receptors forms. Polymorphisms in intronic regions, such as rs179247 and rs12885526, are able to regulate small RNAs production and provide different initiation sites for alternative mRNA generation, which may influence the gene expression or the posttranslational process. On the other hand, the association of *TSHR* gene polymorphisms (rs179247 and rs12885526) with GD and/ or GO susceptibility remains unclear. We aimed to investigate the influence of the genetic profile of two *TSHR* intronic polymorphisms, rs179247 and rs12885526, in the susceptibility and the clinical features of a very well-characterized group of GD and GO patients [5-8].

Graves' disease (GD) is a common autoimmune disorder with manifestations involving the thyroid, orbital connective tissue and other organs. In a 5-year study by Teng *et al*, the incidence of GD has reached 160 cases/100000/year in a mild iodine-deficient Panshan area in China[3]. In recent years, the prevalence of GD tends to increase worldwide. However, the pathogenesi of this condition remains unclear **[9, 5].** Genetic predispositions are considered to be associated with GD besides environmental factors such as high iodine intake, smoking, alcohol consumption, selenium supplementation, vitamin D levels, stress, infections and some drugs. Studies on twins ever indicated that about 80% of GD risk was attributed to genetic factors, though subsequent researchers considered only less than 10% of the risk could be explained by genes. The thyroid stimulating hormone receptor (TSHR), existing on the follicular cell surface of the thyroid, can regulate thyroid growth and stimulate the synthesis of thyroid hormone via thyroid stimulating hormone (TSH). Meanwhile, TSHR is the major autoantigen in GD, and TSHR antibodies are always found to rise in GD patients. GD is characterized by increased lymphocytic filtration of thyroid tissue and elevated levels of TSHR autoantibodies, and the latter can trigger the onset of the disease. Therefore, TSHR gene has long been held to be a susceptibility gene. Studies were initially focused on the single nucleotide polymorphisms (SNPs) in exons of TSHR gene, but most didn't ascertain the relationship between the SNPs and GD [10-14].

MATERIALS AND METHODS

Study population:

The study population consisted of 232 unrelated subjects comprising of 108 Autoimmune Thyroid Disease (AITD) patients and 124 ethnically matched controls of central Indian population were included in this study. In this region Hindu, Muslim and some Sikh peoples are mainly living but most people belong to Hindu religion in this region.

Inclusion and Exclusion criteria for Cases:

Cases included consecutive patients who attended the Department of Medicine, Shyam Shah Medical College and Sanjay Gandhi Memorial Hospital, Rewa, Ayurveda Medical College, Rewa, Ranbaxy pathology Regional collection centre Rewa, District hospital Satna, Shahdol, Sidhi. Autoimmune Thyroid Disease (AITD) was diagnosed in accordance with World Health Organization (WHO Expert committee 2003) criteria. Pregnant women, children under age of 18 years and any patients with Autoimmune Thyroid Disease (AITD) were excluded from the study.

Inclusion and Exclusion criteria for Controls:

Control group composed of non-diabetic healthy individuals that were collected during "Autoimmune Thyroid Disease (AITD) Awareness Camps" organized in urban regions in and around SSMC Rewa and many volunteers were also included to collect control sample. The control subjects were recruited from the regions that from homogenous cluster in Vindhyan region India in accordance with a recent report of genetic landscape of the people of India. The inclusion criteria for control group were as follows:-

ANTHROPOMETRIC AND BIOCHEMICAL MEASUREMENTS

Anthropometry:
Height and Weight were measured in light clothes and without shoes in standing position as per standard guidelines. Body Mass Index (BMI) was calculated as weight in kilograms divided by height in meters squared. Waist circumference was measured in standing position midway between iliac crest and lower costal margin and hip circumference was measured at its maximum waist to hip ratio (WHR) was calculated using waist and hip circumferences. Systolic and diastolic blood pressures were measured twice in the right arm in sitting position after resting for at least 5 minute using a standard sphygmomanometer and the average of the two reading was used.

Biochemical Analysis:
Biochemical parameters related to Autoimmune Thyroid Disease (AITD) were estimated for both cases and controls subjects. Measurement of Serum levels of glucose, Total cholesterol (TC), Triglycerides (TG), HbA1c, High density lipoprotein-cholesterol (HDL-C), Low density lipoprotein-cholesterol (LDL-C), Urea, Uric acid, citrate, TSH, FT3, FT4 and Creatinine were measured based on spectrophotometric method using automated clinical chemistry analyzer Cobas Integra 400 plus (Roche Diagnostics, Mannheim, Germany).

Blood collection and plasma/serum separation:
Venous blood samples were obtained from the subjects after 12 hours of overnight fasting in vacutainers with and without appropriate anti-coagulants. Immediately, plasma and serum from the respective vacutainers were separated by centrifuging the tubes at 1000 rpm for 10 min. at 4˚C

MOLECULAR LABORATORY ANALYSIS

Method for DNA isolation:
Genomic DNA was extracted from whole blood by the modification of salting out procedure described by Miller and coworkers (Miller *et al*. 1988). Frozen blood sample was thawed at room temperature. 0.5 ml. of whole blood sample was suspended in 1.0 ml. of lysis buffer (0.32 M Sucrose, 1 mM Mgcl2, 12 mM Tris and 1% Triton-X-100) in a 1.5 ml. microcentrifuge tubes. This mixture was mixed gently by inverting the tube upside down for 1 min. The mixture was than allowed to stand for 10 min. at room temperature to ensure proper lysis of cells. The mixture was centrifuged at 11,000 rpm for 5 min. at 4˚C to pellet the nuclei. The supernatant was discarded carefully in a jar containing disinfectant, as pellet formed is loosely adhered to the bottom of centrifuge tube. The pellet was resuspended in 0.2 ml. of lysis buffer and recentrifuge at 11,000 rpm for 5 min. The pellet was than dissolved in 0.2 ml. of deionized autoclaved water and mixed thoroughly on vortexer. The mixture was centrifuged at 14,000 rpm for 1 min. at 4˚C. Supernatant was discarded to gain an intact pellet. To the above pellet, 80 µl. of proteinase K buffer (0.375 M Nacl, 0.12 M EDTA, pH 8.0) and 10 µl. of 10% SDS (10% w/v SDS, pH 7.2) was added. Mixture was well frothed with the help of micro tip to allow proper lysis of pelleted nuclei. After digestion was complete, 100 µl. of saturated cold 5M Nacl was added and shaken vigorously for 15 sec. To the above mixture 0.2 ml. of deionized, autoclaved water and 0.4 ml. of phenol-chloroform (4:1 v/v) was added to remove most of the non-nucleic acid organic molecules. Microcentrifuge tube was inverted upside down until the solution turned milky. Phases were separated by centrifuging the above mixture at 12,000 rpm for 10 min. at 4˚C. Aqueous (top) layer was saved and transferred in another microcentrifuge tube. Transferring of any interface layer was avoided. To the aqueous layer, 1 ml. chilled absolute ethanol was added and the tube was inverted several times until the DNA precipitated. DNA precipitates like thread. This was centrifuged at 14,000 rpm for 4 min. at 4˚C to pellet the DNA thread. Supernatant was discarded. The pellet was washed twice with 1 ml. of 70% alcohol. The mixture was again centrifuged at 14,000 rpm for 1 min. 4˚C. Supernatant was discarded and pellet was air dried for 10-20 min. The pelleted DNA was rehydrated in 100-200 µl. of TE buffer pH 7.4 (10 mM Tris-HCL pH 7.4, 1mM EDTA, pH 8.0). DNA was allowed to dissolve overnight at 37˚C before quantization.

Determination of quality and quantity of isolated DNA:
The isolated DNA is to be used for PCR based study. Therefore its suitability for PCR along with its size heterogeneity is among the most important criterion

for purity. As a matter of general practice all DNA preparations were tested for quality and quantity measures, as described in the following paragraphs.

Quantification by UV spectrophotometer:
The isolated genomic DNAs were then tested for purity by measuring their absorbance values at 230 nm, 260 nm, 280 nm and 300 nm using a UV visible spectrophotometer (Systronic, India). A DNA preparation was considered to be good if it had A 260 nm / A 280 nm ratio as approximately 1.8 and A 300 nm was 0.1 or lesser. The absorbance at 260 nm was used to calculate the amount of DNA, using the relationship that double stranded DNA at 50µg/ml concentration has an absorbance= 1.0 at 260 nm.

Agarose Gel Electrophoresis:
Gel electrophoresis of the genomic DNAs was carried out for qualitative estimation of samples prepared. A good DNA preparation appears as single band. A horizontal agarose slab gel electrophoresis apparatus (Bangalore Genei, Bangaore, India) was used. In brief, 4-5 µl of each genomic DNA was loaded on 0.8 agarose (0.8 % w/v, Sigma) containing ethidium bromide solution (0.5 µg/ml) and electrophoresis was done at 80 V in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). Lambda DNA *Eco*RI / *Hind* Ш double digest (Bangalore Genei, Bangalore, India) was used as molecular weight marker after completion of electrophoresis, the DNA bands were visualized and photographed using an UV transilluminator (312 nm) and gel documentation system (Vilber Lourmate, Cedex 1, France) respectively.

Polymorphism screening:
In general, the genomic DNA extracted from peripheral blood of healthy individuals and diseased individuals was subjected to PCR followed by restriction digestion and electrophoresis to genotype both the groups for relevant gene of interest. All the PCRs were carried out in a PTC 200 thermal cycler (MJ Research Inc. USA). PCR is a rapid, inexpensive and simple mean of producing relatively large copy number of DNA molecules from the small amounts of source DNA material, even when the source DNA is of relatively poor quality. Due to the extreme sensitivity, precautions were taken against contamination of the reaction mixture with the trace amounts of DNA, which could serve as an unwanted template. Appropriate negative control was included in each PCR run carried out for each gene, to monitor this contamination of PCR mix to avoid any false positive results. The negative control used for PCR contained whole PCR reaction mix except target DNA which was replaced by HPLC purified water free of RNase, DNase, and any contamination from any other source resembling the gene sequence.

Subsequently restriction enzyme digestion was performed by incubating the double stranded DNA with appropriate amount of restriction enzyme, in its respective buffer as recommended by the supplier and at optimal temperature for that specific enzyme. A typical digestion includes one unit of enzyme per microgram of starting DNA. One enzyme unit is usually defined as the amount of enzyme needed to completely digest one microgram of double stranded DNA in one hour at the appropriate temperature. Their biochemical activity of the restriction enzyme is the hydrolysis of phosphodiester backbone at specific sites in a DNA sequence. Precaution was taken to avoid star activity of restriction enzymes. When DNA is digested with certain restriction enzymes under non-standard conditions, cleavage can occur at sites different from the normal recognition sequence. Such aberrant cutting is called "star activity" which can be due to high pH (>8.0) or low ionic strength, extremely high concentration of enzyme (>100 U/µg of DNA) and presence of organic solvents in the reaction (e.g. ethanol, DMSO).The PCR and restriction digestion conditions were optimized for specific locus of relevant segment of the gene to be studied. The PCR products as well as the digested products were separated on either agarose gel or polyacrylamide gel depending on their size. Gels were stained with ethidium bromide solution (0.5µg/ml) and subsequently visualized and photographed under UV transilluminator.

Detection of TSHR Single Nucleotide Polymorphism:
The TSHR (Thyroid Stimulating Hormone Receptor) Gene has been amplified by PCR. This polymorphism is a functional polymorphism causing change in SNP rs1792472A/G. The oligonucleotide sequence (primers) was designed to amplify the gene wild type gene is 162bp having restriction site for *NlaIII* enzyme cleaves in to 100 and 62 bp fragment.

Primer sequence: The oligonucleotides sequences (primers) used were those described by Liu R *(*Liu R, *et. al. 2012).*
Forward: 5′-ATTTCGGAGGATGGAGAAATA-3′:
Reverse: 5′-GTCTGCGTACTGGGCGGTAA-3′.

PCR Mix:
The PCR was carried out in a final volume of 25 µl, containing 50-100 ng of genomic DNA(4-5 µl), 2.5 µl of 10X *Taq* polymerase buffer (10 mM Tris HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.005% Tween-20, 0.005% NP-40; final concentration 1X; Genetix Biotech Asia Pvt. Ltd.,India), 1 µl of 10 mM dNTPs (Banglore Genei, Bangalore, India), 1 µl of 25 pmol/µl of forward and reverse primers specific for and 1 µl of unit of 1U/ µl Red Taq DNA polymerase (Bangalore genei).

PCR Thermal Program:
After an initial denaturation of 5 min at 95˚C, the samples were subjected to 35 cycles at 95˚C for 1 min, at 58˚C for 45 s, and 72˚C for 45 s, with a final extension of 10 min at 72˚C in a thermal cycler. A 100bp ladder with amplified product has been run under 2.5 % agarose gel electrophoresis.

Restriction digestion by *NlaIII* :
The amplified product size of 162 base pairs (bp) was digested by the specific restriction enzyme, *NlaIII* for 16 h at 37˚C. The mutated genotype was digested, in to 162 bp and 62 bp. The wild-type genotype (AA) was not digested, whereas the mutated homozygous genotype (GG) was cut as a doublet of 100 and 62 bp. The heterozygous genotype (TC) was represented as 2 fragments of 162 and 100 bp whereas 60 bp of DNA fragments are run in the gel. The digestion products were then separated by electrophoresis on a 2.5% agarose gel. The results were documented by digital camera and further saved by gel documentation system.

RESULT

Anthropometric results:
The descriptive data and comparison of anthropometric and biochemical parameters of Autoimmune Thyroid Disease (AITD) patients versus controls are presented in Table no. 4.1. The age, sex, BMI, WHR were the parameters for anthropometric analysis. As expected the Autoimmune Thyroid Disease (AITD) patients had markedly higher levels of weight in men (P<0.0001***) and women (P<0.0001***) resulting BMI of both men (P<0.0001***) and women (P<0.0001***) was significantly associated with Autoimmune Thyroid Disease in vindhyan population. Whereas WHR in Women (P=0.2630) and Men (P=0.2790) were not found significantly different between case and control group (See Table No. 1).

TABLE No-1 Comparison of anthropometric parameters of Autoimmune Thyroid Disease (AITD) patients and healthy controls

Characteristics	*Cases (108)*	*Controls(124)*	*P-value*
n(Men/Women)	108(64/42)	124(76/46)	
Age(years)	52.5±14.3	52.6±14.2	0.9575,ns
Height(m)	161.50±13.3	161.2±13.4	0.8646,ns
Weight (Kg)			
Women	62.5 ±5.7	56.6 ± 4.5	P<0.0001***
Men	68.8±6.6	58.8±6.1	P<0.0001***
BMI (kg/m^2)			
Women	29.6±3.1	26.1 ± 4.3	P<0.0001***
Men	28.6±4.7	25.1± 5.1	P<0.0001***
Waist circumference (cm)			
Women	93.5±6.2	92.6±6.7	0.2918,ns
Men	90.1±7.2	89.2±6.4	0.3145,ns
Hip (cm)			
Women	95.9±2.4	95.5±2.2	0.1868,ns
Men	91.8±4.3	91.2±3.5	0.2428,ns
WHR			
Women	0.98±0.05	0.97±0.08	0.2630,ns
Men	0.98±0.08	0.97±0.06	0.2790,ns

(* denotes level of significant change between case and control)

Biochemical and clinical findings:

Biochemical test performed in the blood sample for following clinical parameters and the findings were tabulated. Statistical analysis was done by using student's t-test and p value suggests the level of significant were changed here. The descriptive data and comparison of biochemical parameters of Autoimmune Thyroid Disease (AITD) patients versus healthy controls are presented in Table no. 4.2. As expected the Autoimmune Thyroid Disease (AITD) patients had markedly higher levels of FT3 (P=0.0192*) and ratio of FT3/FT4 (P<0.0001***) whereas low level TSH (P<0.0001***) were significantly associated to Autoimmune Thyroid Disease (AITD). Whenever, rests of all parameters were not significantly different between patient and healthy population (See Table No. 2).

TABLE No-2 Comparison of Biochemical and clinical findings of Autoimmune Thyroid Disease (AITD) patients and healthy controls

Characteristics	*Cases (108)*	*Controls(124)*	*P-value*
Post-Prandial Glucose (mg/Dl)	118.7±12.4	119.4±11.6	0.6575,ns
HbA1C(%)	5.9±0.7	5.8±0.8	0.3154,ns
HDL-C(mmol/L)	108.8±12.2	109.3±11.6	0.7495,ns
LDL-C (mg/dL)	42.1±2.6	41.8±3.7	0.4818,ns
TG(mg/dL)	125.9±13.2	126.2±12.2	0.8575,ns
Systolic BP (mmHg)	125.4±8.1	124.8±5.7	0.5108,ns
Diastolic BP (mmHg)	87.1±5.8	86.5±6.2	0.4495,ns
Blood Urea(mg/dL)	16.5±1.6	16.8±1.8	0.1838,ns
Urinary Citrate (mmol/24 h)	2.58±0.96	2.62±0.57	0.6958,ns
Serum creatinine (mg/dl)	0.76±0.37	0.71±0.26	0.2305,ns
TSH, mIU/L	1.14±0.40	1.67±0.60	P<0.0001***
FT3, pmol/L	5.95±8.2	4.13±2.4	0.0192*
FT4, pmol/L	16.45±6.2	15.33±3.4	0.0841,ns
FT3/FT4	0.36±0.07	0.26±0.06	P<0.0001***

(* denotes the level of significant change between case and control)

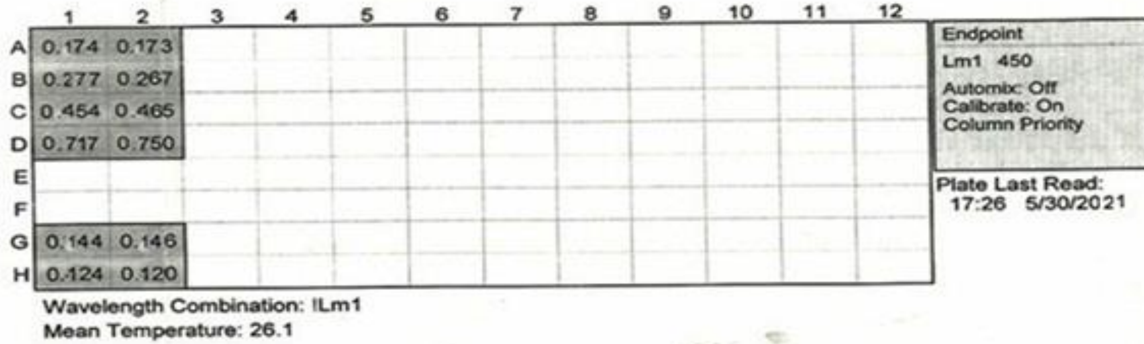
ELISA Analysis of TSH:

TSH is a pituitary hormone associated with Thyroid stimulation. Elevated level of FT3/FT4 in blood responsible for feedback inhibition to releasing of TSH from pituitary gland resulting level of TSH decreased in Autoimmune Thyroid Disease (AITD). Concentration of TSH level in AITD patient (case) and healthy population (control) is depicted in table no.-3, is showing decreased level during AITD and it was statistically significant associated as P<0.0001*** with AITD **(See Table No. 3).** Figure no. 3 (a) and 3 (b) are showing ELISA of TSH result. This is kit based ELISA result reveals elevated TSH level in AITD. In figure no. 3 (a), two columns ELISA analysis having four standards A, B, C, D containing 25, 50, 100, 200 Pg/ml concentration respectively read absorbance at 450 nm. G for Patient (Case) and H for Healthy (control) showing differences in TSH level as 1.14 mIU/L and 1.67 mIU/L respectively.

TABLE No-3 Comparison of Biochemical Factor between Autoimmune Thyroid Disease (AITD) Cases and Healthy Controls:

Characteristics	*Cases (108)*	*Controls(124)*	*P-value*
TSH Level in serum (mIU/L)	1.14±0.40	1.67±0.60	P<0.0001***

(*Denotes level of significant change between malarial cases and healthy controls.)



TSH level in serum (mIU/L)

Exp01:

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.174	0.173										
B	0.277	0.267										
C	0.454	0.465										
D	0.717	0.750										
E												
F												
G	0.144	0.146										
H	0.124	0.120										

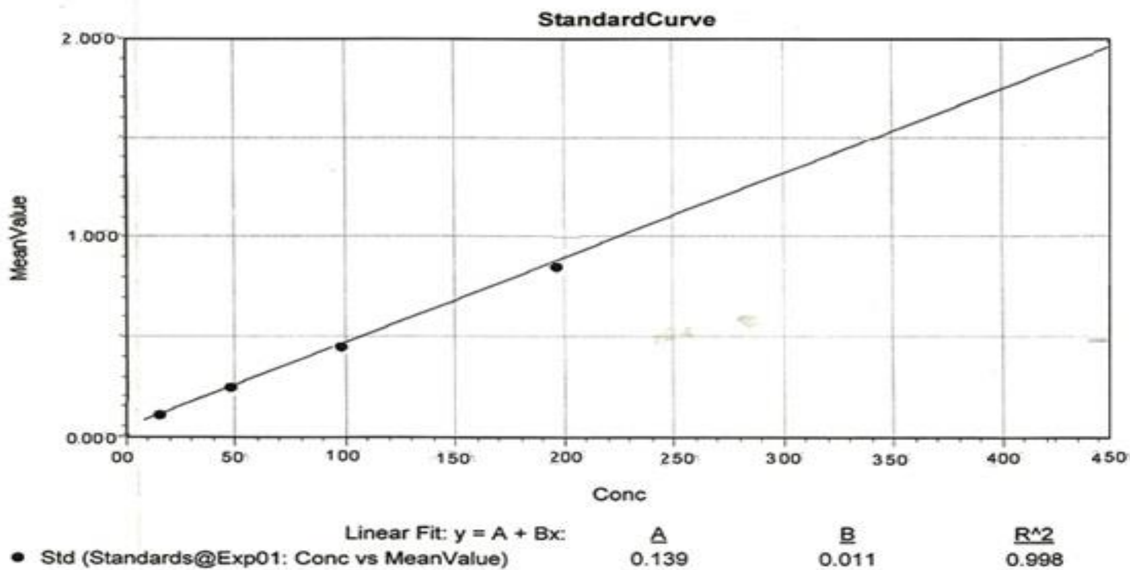
Endpoint
Lm1 450
Automix: Off
Calibrate: On
Column Priority

Plate Last Read:
17:26 5/30/2021

Wavelength Combination: !Lm1
Mean Temperature: 26.1

Sample	Wells	Values	Std.Dev.	CV%	Conc.(mIU/L)
Case	G1	0.144	0.001	0.009	1.67
	G2	0.146			
Control	H1	0.124	0.002	0.022	1.14
	H2	0.120			

Graph No.-1 (a): Result of ELISA, showing TSH Level in both case and control.



Standards (mIU/L)

Sample	Conc	Wells	Value	MeanValue	SD	CV
St01	25.000	A1	0.174	0.173	0.0007	0.004
		A2	0.173			
St02	50.000	B1	0.277	0.272	0.0070	0.257
		B2	0.267			
St03	100.000	C1	0.454	0.459	0.0077	0.016
		C2	0465			
St04	200.000	D1	0.717	0.733	0.0233	0.031
		D2	0.750			

Smallest standard value: 0.173
Largest standard value: 0.733

Graph No.-1 (b); Straight line graph is showing concentration change accordance to absorbance.

Detection of Genetic Polymorphism in TSHR (Thyroid Stimulating Hormone Receptor) Gene:
PCR amplification with specific primers gave 162-bp product which was digested with *NlaIII* enzyme (New England Biolabs, Boverly, MA) for 16 h at 37˚C. The wild-type genotype (AA) was not digested, whereas the mutated homozygous genotype (GG) was cut as a doublet of 100bp and 62bp. The heterozygous genotype (AG) was represented as 3 fragments of 162bp and 100bp and 62bp (Depicted in figure no. 4.1.)

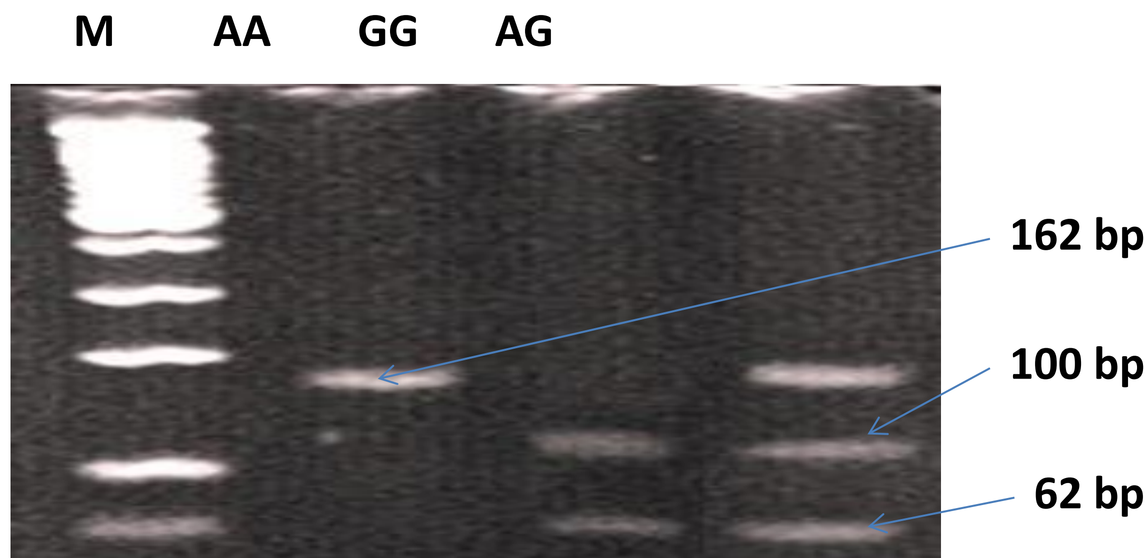


Figure No.-1: Representative gel picture of TSHR polymorphism. Lane M represents 50 bp molecular marker, Lane AA Wild type genotype, Lane AG heterozygous genotype and Lane GG variant genotype

The distribution of the polymorphisms of TSHR was consistent with Hardy- Weinberg equilibrium (HWE) in healthy controls. The observed genotype frequencies, allele frequencies and carriage rates for TSHR polymorphism are depicted in table 5 and table 6 and Graph 1, 2, 3. significant level of change has been seen in overall distribution of TSHR genotypes in Healthy control group as compared to disease group although Healthy control group showed little increase in 'AA' genotype as compared to Patients of Autoimmune Thyroid Disease (66.12% *vs* 50.00%). Similarly, mutant type 'GG' genotype was present in low frequency in Autoimmune Thyroid Disease (AITD) patients group (4.62%) as compared to control group (6.45%) whereas AG genotype frequency higher in disease group as compares to control group (45.37% *vs* 27.41%). Genotype frequency distribution between case and control group was significantly associated with Autoimmune Thyroid Disease (χ^2 = 0.0174*, P= 8.103). In addition, Allele frequency (χ^2 = 3.287, P= 0.0698) and carriage rate (χ^2 = 2.269, P=0.1320) between both groups was not significantly associated to Autoimmune Thyroid Disease.

An odds ratio of AA genotype is 0.5122 which indicates little protective effect whereas an odds ratio of AG genotype is 2.198 of Autoimmune Thyroid Disease (AITD) patients group respectively indicate little or no effect and association of this mutant genotype with the Autoimmune Thyroid Disease (AITD) susceptibility. Overall allele 'A' was found little lower frequency in disease group as compared to HC group whereas allele '*G'* was present in little high frequency in the disease group but the difference is nominal and was not significant (χ^2 = 3.287, P= 0.0698). Carriage rate of allele 'G' was slightly high in Autoimmune Thyroid Disease (AITD) group as compared to healthy control (34.39% Vs 26.58%) whereas carriage rate of allele '*A*' was high in control as compare to disease group and no significant level of change has been seen. The pattern of genotype distribution, allele frequency and carriage rate in disease and control group suggests TSHR polymorphism is significantly associated with Autoimmune Thyroid Disease (AITD) in our population (See Table No. 5 and 6).

TABLE No-4.5 Frequency distribution and association of Genotype, allele frequency and carriage rate of TSHR gene polymorphism in population of Vindhyan region using Chi Square Test

TSHR GENE	CASE N= 108		CONTROL N=124		CHI SQUARE VALUE χ^2 (P Value)
	N	%	N	%	
Genotype					
AA	54	50.00	82	66.12	
AG	49	45.37	34	27.41	8.103 (0.0174*)
GG	5	4.62	8	6.45	
Allele					
A	157	72.68	198	79.83	3.287 (0.0698, ns)
G	59	27.31	50	20.16	
Carriage Rate					
A	103	65.60	116	73.41	2.269 (0.1320, ns)
G	54	34.39	42	26.58	

(- denotes the level of significant association between case and control.)*

(N – Number of individuals in study group.)

(% - Genotype allele frequency and carriage rate expressed in percentage.)

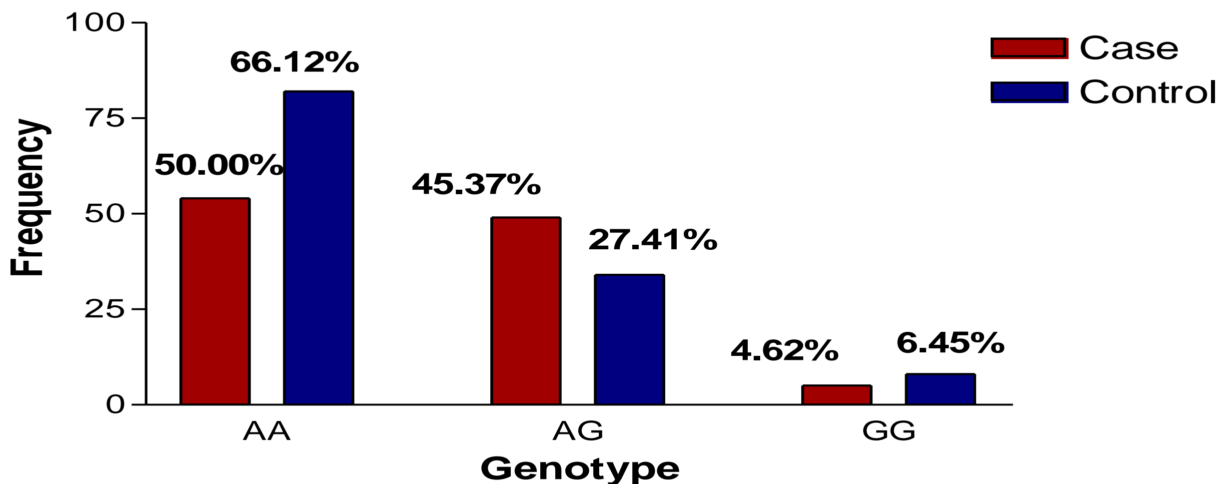
TABLE No-4.6 Fisher Exact Test values of TSHR gene polymorphism

TSHR GENE	CASE N= 108		CONTROL N=124		P Value	Odds Ratio (95% confidence interval)
	N	%	N	%		
Genotype						
AA	54	50.00	82	66.12	0.0161*	0.5122 (0.3015 to 0.8700)
AG	49	45.37	34	27.41	0.0059**	2.198 (1.272 to 3.799)
GG	5	4.62	8	6.45	0.5825ns	0.7039 (0.2232 to 2.220)
Allele						
A	157	72.68	198	79.83	0.0792ns	0.6720 (0.4366 to 1.034)
G	59	27.31	50	20.16		1.488 (0.9669 to 2.290)
Carriage Rate						
A						
G	103	65.60	116	73.41	0.1431ns	0.6906 (0.4261 to 1.119)
	54	34.39	42	26.58		1.448 (0.8935 to 2.347)

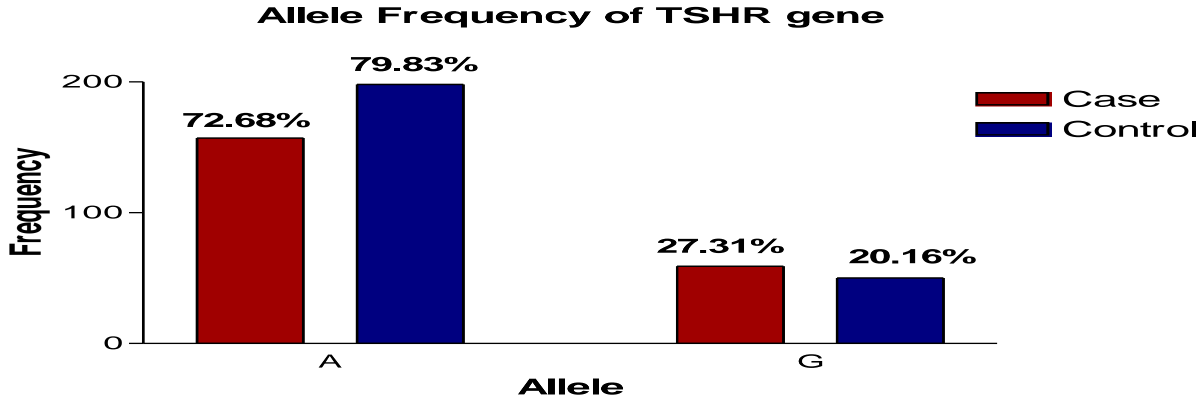
(* - denotes the level of significant association between case and control.)

(N – Number of individuals in study group.)

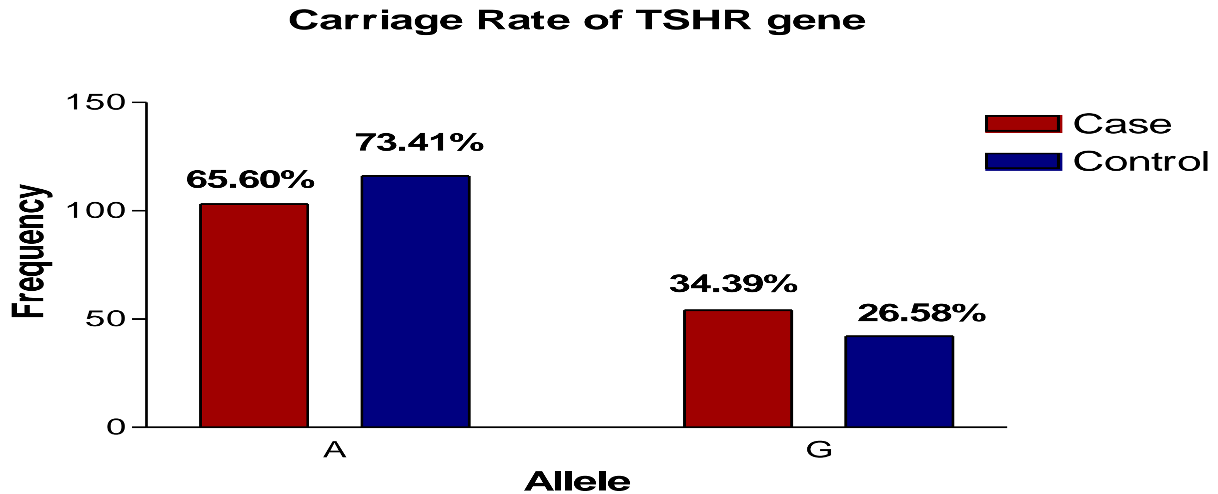
(% - Genotype allele frequency and carriage rate expressed in percentage.)



Graph No.-2: Genotype Frequency of TSHR gene.



Graph No.-3: Allele Frequency of TSHR gene



Graph No.-4: Carriage rate of TSHR gene

DISCUSSION

Thyroid hormones are crucial for organism development and homeostasis. In humans, untreated congenital hypothyroidism due to thyroid agenesis inevitably leads to cretinism, which comprises irreversible brain dysfunction and dwarfism. Elucidating how the thyroid gland – the only source of thyroid hormones in the body – develops is thus, key for understanding and treating thyroid dysgenesis, and for generating thyroid cells in vitro that might be used for cell-based therapies **[15].** The principal mechanisms involved in thyroid organogenesis and functional differentiation, highlighting how the thyroid forerunner evolved from the endostyle in protochordates to the endocrine gland found in vertebrates. Autoimmune thyroid diseases (AITD), including Graves' disease (GD) and Hashimoto's thyroiditis, arise due to complex interactions between environmental and genetic factors. Each is presenting with distinct clinical features. Significant progress has been made in our understanding of the mechanisms leading to AITD. Because of the complex nature of AITD, caused by their polygenic nature and a complex mode of inheritance, there are still more questions to be answered than answers that can be given, especially about the nature of Hashimoto's thyroiditis **[16-18].**
The genetic contribution to AITD will hold one of the keys to understanding disease pathogenesis and developing improved treatments. Common HT and GD genes have been identified, as well as genes that are characteristic for only one of those diseases. In this review, we summarize the findings on the genetic susceptibility to AITD focusing on emerging mechanisms of susceptibility **[19].** Genetics play a prominent role in both determination of thyroid hormone and thyrotropin (TSH) concentrations, and susceptibility to autoimmune thyroid disease. Heritability studies have suggested that up to 67% of circulating thyroid hormone and TSH concentrations

are genetically determined, suggesting a genetic basis for narrow intra-individual variation in levels, perhaps a genetic 'set point'. The search for the genes responsible has revealed several candidates, including the genes for phosphodiesterase 8B, iodothyronine deiodinase 1, F-actin-capping protein subunit beta and the TSH receptor; however, each of these only contributes a small amount to the variability of hormone concentrations, suggesting that further genes and mechanisms of genetic influence are yet to be discovered. Some genes known to influence thyroid function, including iodothyronine deiodinase 2 and the TSH receptor, have been shown to influence a wide range of clinical and developmental phenotypes from bone health to neurological development and longevity; such observations will help us understand the complex action of thyroid hormones on individual tissues **[20].** Finally, autoimmune thyroid disease commonly runs in families, and the search for genes which increase susceptibility has identified several good candidates, particularly those involved in immune regulation and thyroid function. However, these genes alone account for only a small percentage of the current prevalence of these disorders. Although the advancement of genetic technology has led to many significant findings in the last decade or two, it is clear that we are only just beginning to understand the role of genetics in thyroid function and disease [21-24].

Our statistical data from anthropometric and biochemical parameters in Autoimmune Thyroid Disease (AITD) patients and controls are significantly different in BMI. The age, sex, BMI, WHR were the parameters for anthropometric analysis. As expected the Autoimmune Thyroid Disease (AITD) patients had markedly higher levels of weight in men (P<0.0001***) and women (P<0.0001***) resulting BMI of both men (P<0.0001***) and women (P<0.0001***) was significantly associated with Autoimmune Thyroid Disease in vindhyan population. Whereas WHR in Women (P=0.2630) and Men (P=0.2790) were not found significantly different between case and control group. Thus Biochemical test performed in the blood sample for following clinical parameters and the findings were tabulated. Statistical analysis was done by using student's t-test and p value suggests the level of significant were changed here. The descriptive data from comparison of biochemical parameters of Autoimmune Thyroid Disease (AITD) patients versus healthy controls are significantly associated to Autoimmune Thyroid Disease (AITD) as higher levels of FT3 (P=0.0192*) and ratio of FT3/FT4 (P<0.0001***) whereas low level TSH (P<0.0001***). Whenever, rests of all parameters were not significantly different between patient and healthy population.

The relationship between thyroid stimulating hormone (TSH) and obesity has been widely discussed. Serum free triiodothyronine (fT3) and fT3/free thyroxine (fT4) ratio (fT3/fT4) were positively associated with body mass index (BMI) (P < 0.001), while there was a negative relationship between fT4 and BMI (P < 0.001) according to multivariable regression analysis adjusted for age and sex. Associations between thyroid hormone concentrations and markers of blood pressure, and lipid and glucose metabolism were identified after adjustment for age, sex, and BMI, with TSH being negatively associated with fasting blood glucose [25]. fT3 was positively associated with systolic blood pressure and low-density lipoprotein-cholesterol, while fT4 was positively associated with diastolic blood pressure, FBG, and high-density lipoprotein-cholesterol (HDL-C), and negatively associated with hemoglobin A1c (HbA1c) and triglyceride. Finally, fT3/fT4 was positively associated with HbA1c and triglyceride, and negatively associated with HDL-C. Overweight or obese participants had a high serum concentration of fT3, high fT3/fT4 ratio, and a low concentration of fT4. Underweight participants had high concentrations of fT4 and low concentrations of fT3. Thus, relationships between thyroid hormones and metabolic risk markers were identified which suggest that thyroid function might be one factor that influences body weight and the co-morbidities of obesity [26].

Our result from ELISA suggested that Elevated level of FT3/FT4 in blood responsible for feedback inhibition to releasing of TSH from pituitary gland resulting level of TSH decreased in Autoimmune Thyroid Disease (AITD). Concentration of TSH level in AITD patient (case) and healthy population (control) was significantly different and it was associated as P<0.0001*** with AITD. ELISA of TSH was a kit based result reveals elevated TSH level in AITD. Two columns ELISA analysis having four standards A, B, C, D containing 25, 50, 100, 200 Pg/ml concentration respectively read absorbance at 450 nm. G for Patient (Case) and H for Healthy (control)

showing differences in TSH level as 1.14 mIU/L and 1.67 mIU/L respectively. Our research on TSHR polymorphism revealed association with AITD. The PCR amplification with specific primers gave 162-bp product which was digested with *NlaIII* enzyme for 16 h at 37˚C. The wild-type genotype (AA) was not digested, whereas the mutated homozygous genotype (GG) was cut as a doublet of 100bp and 62bp. The heterozygous genotype (AG) was represented as 3 fragments of 162bp and 100bp and 62bp. The distribution of the polymorphisms of TSHR was consistent with Hardy- Weinberg equilibrium (HWE) in healthy controls [27, 21].

Significant level of change has been seen in overall distribution of TSHR genotypes in Healthy control group as compared to disease group although Healthy control group showed little increase in 'AA' genotype as compared to Patients of Autoimmune Thyroid Disease (66.12% *vs* 50.00%). Similarly, mutant type 'GG' genotype was present in low frequency in Autoimmune Thyroid Disease (AITD) patients group (4.62%) as compared to control group (6.45%) whereas AG genotype frequency higher in disease group as compares to control group (45.37% *vs* 27.41%). Genotype frequency distribution between case and control group was significantly associated with Autoimmune Thyroid Disease (χ^2 = 0.0174*, P= 8.103). In addition, Allele frequency (χ^2 = 3.287, P= 0.0698) and carriage rate (χ^2 = 2.269, P=0.1320) between both groups was not significantly associated to Autoimmune Thyroid Disease. An odds ratio of AA genotype is 0.5122 which indicates little protective effect whereas an odds ratio of AG genotype is 2.198 of Autoimmune Thyroid Disease (AITD) patients group respectively indicate little or no effect and association of this mutant genotype with the Autoimmune Thyroid Disease (AITD) susceptibility. Overall allele 'A' was found little lower frequency in disease group as compared to HC group whereas allele '*G'* was present in little high frequency in the disease group but the difference is nominal and was not significant (χ^2 = 3.287, P= 0.0698). Carriage rate of allele 'G' was slightly high in Autoimmune Thyroid Disease (AITD) group as compared to healthy control (34.39% Vs 26.58%) whereas carriage rate of allele '*A*' was high in control as compare to disease group and no significant level of change has been seen. The pattern of genotype distribution, allele frequency and carriage rate in disease and control group suggests TSHR polymorphism is significantly associated with Autoimmune Thyroid Disease (AITD) in our population [28].

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