IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

"Association study of Genetic polymorphism of *CTLA4* gene with Autoimmune Thyroid Disease (AITD): A Case-Control Study of Vindhyan Population"

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Abstract:

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is a surface protein on T cells, that has an inhibitory effect on the host immune reaction and prevents overreaction of the immune system. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) has an important role in homeostasis and negative regulation of immune responses, and is therefore considered to be a key element in the development of autoimmune diseases. Genotyping was performed using the tetra-primer amplification refractory mutation system polymerase chain reaction technique. Full-length human *CTLA4* gene spans 6.1kb DNA, with 4 exons and 3 introns. This small region is very polymorphic as manifested by the enrichment of many exonic, intronic, and promoter single nucleotide polymorphisms (SNPs). Although the mechanisms underlying CTLA4 mediated GD developments are yet to be fully addressed, elucidation of its genetic predisposition to GD, however, may offer some important clues.

Significant level of change has been seen in overall distribution of CTLA4 genotypes in Healthy control group as compared to disease group although Healthy control group showed little increase in 'TT' genotype as compared to Patients of Nephrolithiatic (69.44% *vs* 56.25%). Similarly, mutant type 'CC' genotype was present in low frequency in Nephrolithiatic patients group 3.75% and also in control group 4.44% (χ^2 = 0.0243*), P= 7.436). An odds ratio of TT genotype is 0.5657 which indicates little protective effect whereas an odds ratio of TC genotype is 1.887 of Nephrolithiatic patients group respectively indicate little or no effect and association of this mutant genotype with the Nephrolithiatic susceptibility. Overall allele 'C' was found little lower frequency in disease group as compared to HC group whereas allele '*T'* was present in little high frequency in the disease group but the difference is nominal and was not significant (χ^2 = 0.0437*, P=4.069).

Keywords: CTLA4 gene, GD, Allele,

Introduction:

Autoimmune thyroid disease (AITD), including Graves disease (GD) and Hashimoto disease (HD), is an organ-specific autoimmune disease characterized by the presence of autoantibodies and T cell-mediated autoimmunity against self-antigens. Both GD and HD involve similar genetic background and additional environmental and hormonal factors. Antibody-mediated thyroid stimulation prominently occurs in GD, whereas lymphocyte- and cytokine-mediated thyroid apoptosis predominates in HD, but overlap may occur. They are the most prevalent autoimmune endocrinological diseases in children and adolescents, and are estimated to affect approximately 1% of the general population. Although the exact etiology has not been fully clarified, the current hypothesis is that a complex interplay between genetic and environmental factors causes AITD **[1-3].**

AITD has been found to be clustered in families. The risk ratio for a female sibling (λs) of a proband with GD is 15–20. The concordance rate of GD is 20–35% in monozygotic twins, but only 3–7% in dizygotic twins. Twin studies also reveal that genetic factors contribute to about 75% of the development of AITD. These observations strongly suggest that genetic factors are important in the pathogenesis of AITD. The most important gene involved in AITD is the HLA-DR locus. The CTLA4 gene encodes a transmembrane regulatory protein, cytotoxic T-lymphocyteassociated protein 4, which is expressed on activated T cells and negatively regulates their function **[4].** CTLA4 competes with CD 28 binding with its ligand B7 on antigen presenting cells, raises the threshold of T cell activation, increases T cell motility and overrides T cell receptor induced stop signal required for stable conjugate formation between T cells and antigen presenting cells. Several polymorphic sites in the gene, including C>T polymorphism in the promoter –318 (rs5742909), A>G polymorphism in exon 1 +49A/G (rs231775), microsatellite (AT)n repeat in the 3'-untranslated region (UTR), and three single nucleotide polymorphisms (SNPs) in the 6.1-kb 3' non-coding region, CT60 (rs3087243G>A), JO31 and JO30, are associated with organ-specific autoimmune disorders in several racial groups. Among them, +49A/G and CT60 are the most widely investigated markers of autoimmune diseases. These two polymorphisms are associated with thyroid antibody production, GD relapse, Graves ophthalmopathy, and susceptibility to GD and HD **[5-7].**

The thyroid gland is important in the human body because of its ability to produce the hormones triiodothyronine (T3) and tetraiodothyronine (T4), necessaries for appropriate energy levels, and an active life.Thyroid hormones are important in several physiological processes such as metabolism , normal growth development, maintaining the initial level of phospholipids in cell membranes , fatty acids composition of the lipids and any imbalance in their level could lead to a wide range of clinical conditions. Autoimmune thyroid disease (AITD) is the most prevalent autoimmune disorder, related to cellular and humoral immune responses targeted at the thyroid gland and affecting many populations. Hyperthyroidism is the condition caused by overproduction of thyroid hormone. The prevalence's are varied, depending on different ethnic, geographic area, and the criteria for diagnosis **[9].** In the past, this disorder had been found in the young to middle-age group whereas it is now well recognized in the elder group. The major identified AITD susceptibility genes are classified in two functional groups: 1) immune regulatory genes: cytotoxic T lymphocyte-associated antigen 4 (CTLA-4); protein tyrosine

phosphatase, nonreceptor type 22 (PTPN22); interleukin 2 receptor (IL-2R); and 2) thyroidspecific genes: the thyroglobulin gene (TG) and the thyrotropin receptor gene (TSHR). CTLA-4 is a member of the immunoglobulin gene superfamily and a negative regulator of T cell responses that is associated with immune tolerance. CTLA-4 is expressed on the surface of T cells mainly in the form of a dimer, and when it interacts with its cognate ligands, this induces inhibitory signals which terminate T cell activation and proliferation. Polymorphisms in CTLA-4 may alter its functionality such that the activation of T cells cannot be inhibited, resulting in a loss of immune tolerance and the occurrence of autoimmunity, making it vital that normal CTLA-4 activity be maintained. CTLA-4 is a major susceptibility gene associated with autoimmune thyroid disease **[10-13].**

Graves' disease (GD) is an autoimmune thyroid disease with a 0.5% rate of prevalence in the general population. It is characterized by the presence of thyroid-stimulating hormone (TSH) receptor antibodies leading to hyperthyroidism and goiter. The exact etiology of GD remains unknown; however, it is believed that genetic polymorphisms and environmental factors are both involved in pathogenesis. It has now been established that the thyroid gland in patients with GD is infiltrated by lymphocytes, predominantly T lymphocytes; a T lymphocyte immune regulating gene cluster is located in the 2q33 gene region. The cytotoxic T-lymphocyte antigen 4 (*CTLA4*) gene, residing in human chromosome 2q33, encodes a key negative regulator of T-cell activation and proliferation during the immune response and thereby may influence T-cell mediated autoimmune diseases such as GD. Therefore, the *CTLA4* gene is a functional candidate genetic marker for studying GD **[14-17].**

Materials & 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* III 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 (0.5 µg/ml) and subsequently visualized and photographed under UV transilluminator.

Detection of CTLA4 Polymorphism:

CTLA4 +49A/G gene has been amplified by PCR. The Primer sequences oligonucleotide sequence (primers) was designed to amplify the gene wild type gene is lack of restriction site for *MseI* enzyme but mutant allele contains a restriction site.

PCR Primer: The oligonucleotides sequences (primers) used were those described by **Sousa MM**, ***(Sousa MM**, **et. al. 2021).***

Forward primer- 5′-AAATGAATTGGACTGGATGGT-3′

Reverse primer- 5′-TTACGAGAAAGGAAGCCGTG-3′

PCR Mix:

The PCR was carried out in a final volume of 25 µl, containing 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 (Bangalre genei).

PCR Thermal Program:

After an initial denaturation of 5 min at 94˚C, the samples were subjected to 35 cycles at 94˚C for 1 min, at 55˚C for 40 s, and 72˚C for 40 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 1 % agarose gel electrophoresis. 192bp product will be generated after PCR.

Restriction Digestion by *MseI*:

Restriction Digestion The 238-bp product was digested with *MseI* enzyme (New England Biolabs, overly, MA) for 16 h at 37˚C. The PCR products when digested by restriction enzyme and wild type allele 192bp segment which were generated by PCR but the mutant allele shows 98 and 41 bp segments. The product sizes are Wild type homozygote, 192 bp; mutant homozygote, 192 and 41 bp; and heterozygote, 192, 98, and 41 bp respectively. Samples will analyzed by electrophoresis using 1.3% agarose gels to analyze the genotype pattern of the gene.

Results:

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)

Detection of Genetic Polymorphism in CTLA4 gene:

The genetic polymorphism in CTLA4 gene create restriction site for *MseI.* The PCR products when digested by restriction enzyme and wild type allele 192bp segment which were generated by PCR but the mutant allele shows 98bp and 41bp segments. The product sizes are Wild type homozygote, 192bp; mutant homozygote, 98bp and 41bp; and heterozygote, 192, 98, and 41bp respectively **(Depicted in figure no. 2.)**

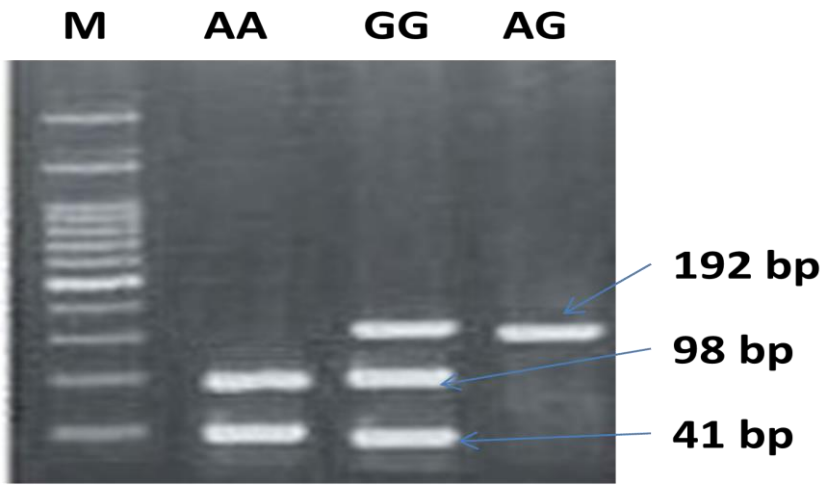


Figure No.-4.2: Representative gel picture of CTLA4 polymorphism. Lane M represents 50 bp molecular marker, Lane AA Wild type genotype, Lane AG heterozygous genotype and Lane GG variant genotype.

The distribution of polymorphic genotype was strongly under HWE. The observed genotype frequencies, allele frequencies and carriage rates for *CTLA4* +49A/G polymorphism are depicted in table 6 and table 7 and graph no. 4, 5, 6. AA genotype frequency was high in HC group in compare to disease group (67.74% *vs* 48.14%) thus GG genotype too (5.64% *vs* 4.62%) but AG frequency high in disease group in compare to HC groupe (47.22 % *vs* 26.61%). Overall distribution of *CTLA4* +49A/G genotypes was significantly different in healthy control group as compared to disease group (χ^2=10.67, P=0.0048**). HC group showed a increased of mutant 'GG' genotype as compared to Patients of Autoimmune Thyroid Disease (5.64% *vs.* 4.62%). Similarly, wild type '*AA*' genotype was present in significantly high frequency in HC as compared to Autoimmune Thyroid Disease (AITD) patients group (67.74% *vs.* 48.14%). An odds ratio of 0.4422 in Autoimmune Thyroid Disease (AITD) group for 'AA*'* genotype indicated a protective effect in our population whereas an odds ratio of AG (2.467) and Mutant GG (0.8114) were not protective for Autoimmune Thyroid Disease (AITD). The patients group respectively indicated a positive association of this wild type genotype with the disease, A is also significantly different but may be not protective because of odds ratio of 0.5942. Overall allele 'A' was found to be in significantly low frequency in disease group as compared to HC group whereas allele '*G'* was present in significantly high frequency in the healthy control group (χ^2 =5.578, P= 0.0182*). Carriage rate of allele 'A' was high in HC group whereas carriage rate of allele 'G' was high in disease group (χ^2 =6.180 P=0.0129*) but the values were not significant. The pattern of genotype and allele distribution in disease and control group suggested a significant association of *CTLA4* +49A/G to allele 'G' carriage (carriage of 'GG') in Susceptibility to Autoimmune Thyroid Disease (AITD) and not show the protective effect **(See Table No. 7 and 8).**

TABLE No-7

Frequency distribution and association of Genotype, allele frequency and carriage rate of CTLA4 gene polymorphism in population of Vindhyan region using Chi Square Test

CTLA4 GENE	CASE N= 108 N	%	CONTROL N=124 N	%	CHI SQUARE VALUE χ^2 (P Value)
Genotype					
AA	52	48.14	84	67.74	
AG	51	47.22	33	26.61	10.67 (0.0048**)
GG	5	4.62	7	5.64	
Allele					
A	155	71.75	201	81.04	
G	61	28.24	47	18.95	5.578 (0.0182*)
Carriage Rate					
A	103	64.77	117	74.52	
G	56	35.22	40	25.47	3.545 (0.0597, ns)

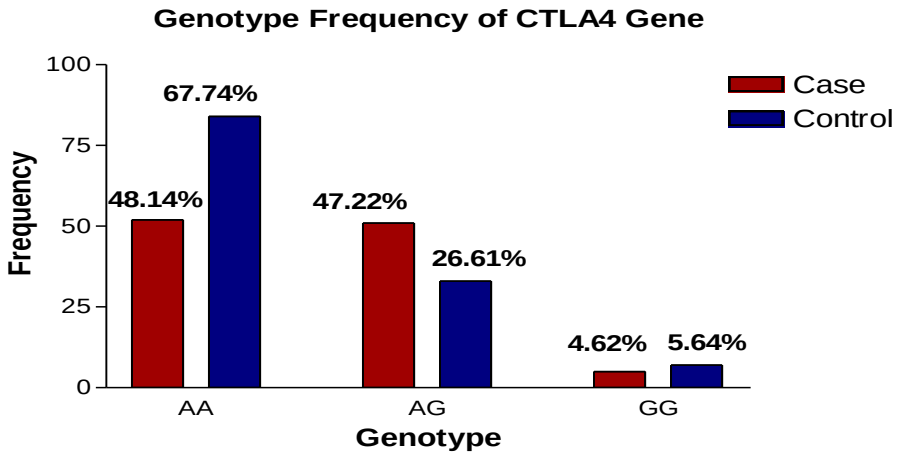
(- denotes the level of significant association between case and control.)*
(N – Number of individuals in study group.)
(% - Genotype allele frequency and carriage rate expressed in percentage.)

TABLE No-4.8

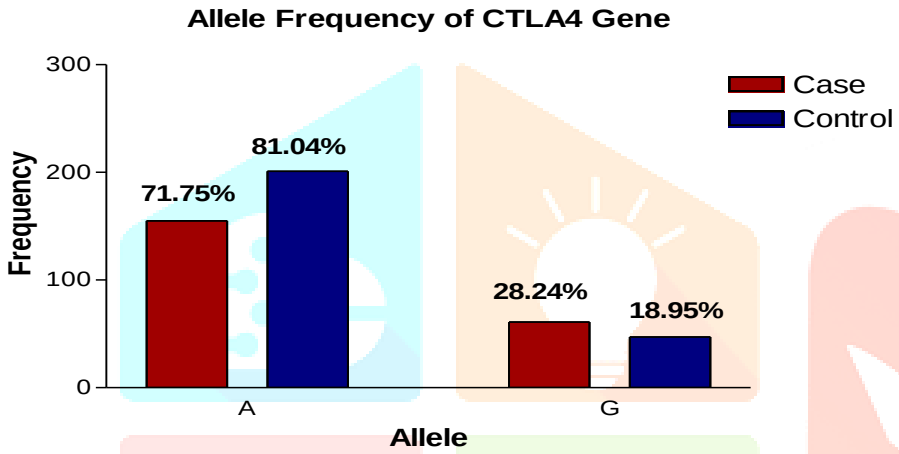
Fisher Exact Test values of CTLA4 gene polymorphism

CTLA4 GENE	CASE N= 108 N	%	CONTROL N=124 N	%	P Value	Odds Ratio (95% confidence interval)
Genotype						
AA	52	48.14	84	67.74	0.0032**	0.4422 (0.2594 to 0.7537)
AG	51	47.22	33	26.61	0.0016**	2.467 (1.425 to 4.273)
GG	5	4.62	7	5.64	0.7752ns	0.8114 (0.2498 to 2.635)
Allele						
A	155	71.75	201	81.04	0.0207*	0.5942 (0.3848 to 0.9174)
G	61	28.24	47	18.95		1.683 (1.090 to 2.599)
Carriage Rate						
A	103	64.77	117	74.52		0.6288 (0.3873 to 1.021)
G	56	35.22	40	25.47	0.0670ns	1.590 (0.9795 to 2.582)

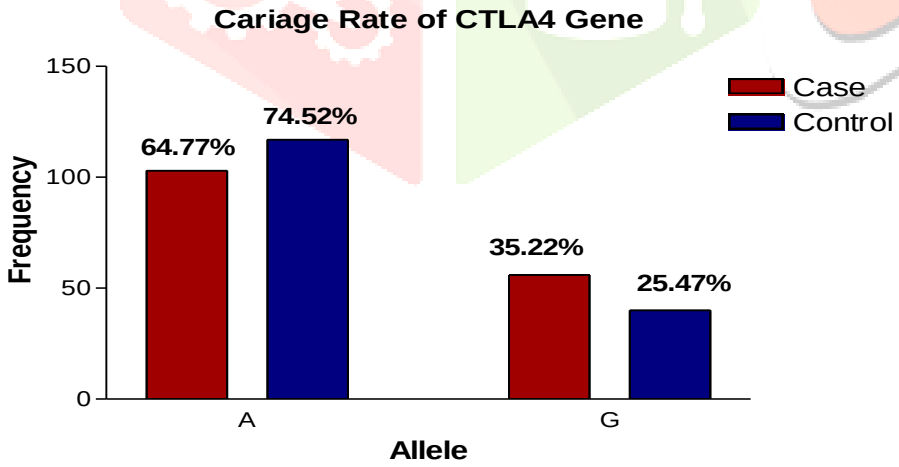
(- denotes the level of significant association between case and control.)*
(N – Number of individuals in study group.)
(% - Genotype allele frequency and carriage rate expressed in percentage.)



Graph No.-4.5: Genotype Frequency of CTLA4 gene



Graph No.-4.6: Allele Frequency of CTLA4 gene



Graph No.-4.7: Carriage rate of CTLA4 gene

Discussion:

Autoimmune thyroid diseases (AITDs) which include Graves' disease (GD) and Hashimoto's thyroiditis (HT) as well as type-1diabetes (T1D) are common autoimmune disorders in children. Many genes are involved in the modulation of the immune system and their polymorphisms might predispose to autoimmune diseases

development. According to the literature genes encoding IL2RA (alpha subunit of Interleukin 2 receptor), IFIH1 (Interferon induced with helicase C domain 1) and CTLA-4 (cytotoxic T cell antigen 4) might be associated with autoimmune diseases pathogenesis. The aim of the study was to assess the association of chosen single nucleotide polymorphisms (SNPs) of IL2RA, IFIH1, and CTLA-4 genes in the group of Polish children with AITDs and in children with T1D **[18, 20].** Single nucleotide polymorphisms (SNPs) in the IL2RA region (rs7093069), IFIH1 region (rs1990760) and CTLA-4 region (rs231775) in group of Polish children and adolescents with type 1 diabetes (*n* = 194) and autoimmune thyroid diseases (GD *n* = 170, HT *n* = 81) and healthy age and sex matched controls for comparison (*n* = 110). There were significant differences observed between T1D patients and control group in alleles of IL2RA (rs7093069 T > C) and CTLA-4 (rs231775G > A). In addition, the study revealed T/T genotype at the IL2RA locus (rs7093069) and G/G genotype at the CTLA-4 locus (rs231775) to be statistically significant more frequent in children with T1D. Moreover, genotypes C/T and T/T at the IFIH1 locus (rs1990760) were significantly more frequent in patients with T1D than in controls. We observed no significant differences between AITD patients and a control group in analyzed SNPs. each allele T of rs7093069 SNP at the IL2RA locus and G allele of rs231775 SNP at the CTLA-4 locus as well as C/T and T/T genotypes of rs1990760 SNP at the IFIH1 locus are predisposing in terms of T1D development. Thereby, we confirmed that IL2RA, IFIH1, and CTLA-4 gene locus have a role in T1D susceptibility. The analysis of selected SNPs revealed no association with AITDs in a group of Polish children and adolescents **[19-22].**

Our research on the genetic polymorphism in CTLA4 gene create restriction site for *MseI*. The PCR products when digested by restriction enzyme and wild type allele 192bp segment which were generated by PCR but the mutant allele shows 98bp and 41bp segments. The product sizes are Wild type homozygote, 192bp; mutant homozygote, 98bp and 41bp; and heterozygote, 192, 98, and 41bp respectively. The distribution of polymorphic genotype was strongly under HWE. The observed genotype frequencies, allele frequencies and carriage rates for *CTLA4* +49A/G polymorphism are depicted in table 6 and table 7 and graph no. 4, 5, 6. AA genotype frequency was high in HC group in compare to disease group (67.74% *vs* 48.14%) thus GG genotype too (5.64% *vs* 4.62%) but AG frequency high in disease group in compare to HC groupe (47.22 % *vs* 26.61%). Overall distribution of *CTLA4* +49A/G genotypes was significantly different in healthy control group as compared to disease group (χ^2=10.67, P=0.0048**). HC group showed a increased of mutant 'GG' genotype as compared to Patients of Autoimmune Thyroid Disease (5.64% *vs.* 4.62%). Similarly, wild type '*AA*' genotype was present in significantly high frequency in HC as compared to Autoimmune Thyroid Disease (AITD) patients group (67.74% *vs.* 48.14%). An odds ratio of 0.4422 in Autoimmune Thyroid Disease (AITD) group for '*AA'* genotype indicated a protective effect in our population whereas an odds ratio of AG (2.467) and Mutant GG (0.8114) were not protective for Autoimmune Thyroid Disease (AITD). The patients group respectively indicated a positive association of this wild type genotype with the disease, A is also significantly different but may be not protective because of odds ratio of 0.5942. Overall allele 'A' was found to be in significantly low frequency in disease group as compared to HC group whereas allele '*G'* was present in significantly high frequency in the healthy control group (χ^2 =5.578, P= 0.0182*). Carriage rate of allele 'A' was high in HC group whereas carriage rate of allele 'G' was high in disease

group (χ^2 =6.180 P=0.0129*) but the values were not significant. .The pattern of genotype and allele distribution in disease and control group suggested a significant association of *CTLA4* +49A/G to allele 'G' carriage (carriage of 'GG') in Susceptibility to Autoimmune Thyroid Disease (AITD) and not show the protective effect.

The association between cytotoxic T-lymphocyte antigen-4 (*CTLA-4*) gene polymorphism and type 1 diabetes mellitus (T1D) in some ethnic populations, and a lack of association in other populations. Differences in the contribution of the genetic background of T1D onset are age dependent. We conducted a case-control study of a T1D Brazilian population, in which a possible association of rs231775 (+49A/G) and rs5792909 (-318C/T) polymorphisms in *CTLA-4* with T1D was evaluated **[8, 23].** These polymorphisms were genotyped in 150 childhood-onset (age 14 years old) and 150 adult-onset (age >18 years) patients with diabetes and non-diabetic healthy individuals. PCR-restriction fragment length polymorphism (rs5792909) and TaqMan® fluorescent probe (rs231775) methodologies were used for genotyping. The polymorphisms were in Hardy-Weinberg equilibrium. There was no difference in genotype and allele frequency between the patients with T1D and non-diabetic controls. The frequencies for childhood-T1D and adulthood-T1D, for the rs231775 G-allele (95% CI) were 39.0% and 37.3%, and for the T-allele of the rs5792909 polymorphism they were 5.0% (3–7%) and 2.7% (1–4%), respectively **[24].** A case/control study to assess the impact of SNP rs3087243 and rs231775 within the *CTLA4* gene was similar to our work. The frequency of G allele for rs3087243 and rs231775 was observed to be significantly higher in subjects with GD than in control subjects (p = 0.005 and p = 0.000, respectively). After logistic regression analysis, a significant association was detected between SNP rs3087243 and GD in the additive and recessive models. Similarly, association for the SNP rs231775 could also be detected in the additive model, dominant model and recessive model. A meta-analysis, including 27 published datasets along with the current dataset, was performed to further confirm the association **[25].**

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is a surface protein on T cells, that has an inhibitory effect on the host immune reaction and prevents overreaction of the immune system. Because the functional single-nucleotide polymorphism (SNP) rs231775 of the CTLA-4 gene is associated with autoimmune diseases and because of the critical role of the immune reaction in sepsis, we intended to examine the effect of this polymorphism on survival in patients with sepsis. 644 septic adult Caucasian patients were prospectively enrolled in this study. Patients were followed up for 90 days. Mortality risk within this period was defined as primary outcome parameter **[7, 9].** A significantly lower 90 day mortality risk among GG homozygous patients (n = 101) than among A allele carriers (n = 543; 22% and 32%, respectively; p = 0.03565). Furthermore, the CTLA-4 rs231775 GG genotype remained a significant covariate for 90-day mortality risk after controlling for confounders in the multivariate Cox regression analysis (hazard ratio: 0.624; 95% CI: 0.399–0.975; p = 0.03858). The first evidence for CTLA-4 rs231775 as a prognostic variable for the survival of patients with sepsis and emphasizes the need for further research to reveal potential functional associations between CTLA-4 and the immune pathophysiology of sepsis GD **[24-26].**

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