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Original article

ASSOCIATION OF PTPN22 POLYMORPHISM AND ITS CORRELATION WITH GRAVES' DISEASE PATIENTS

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ABSTRACT

Background- The etiology of the autoimmune thyroid diseases (AITDs), Graves' disease (GD) is largely unknown. However, genetic susceptibility is believed to play a major role.
The lymphoid tyrosine phosphatase (LYP), encoded by the protein tyrosine phosphatase-22 (PTPN22) gene, is a powerful inhibitor of T cell activation. Recently, a single-nucleotide polymorphism (SNP), encoding a functional arginine to tryptophan residue change at 1 PTPN22 has been shown to be associated with GD and other autoimmune diseases. Aim - We aimed to analyze whether SNP of PTPN22 gene has any association with GD in Kumaon population. Method- We have used a polymerase chain reaction (PCR)-restriction fragment (XcmI) assay to examine genotypes polymorphism in 50 unrelated patients with AITD and 50 controls. Results -Among the patients with GD, the frequencies of PTPN22 CC, CT, and TT genotypes were 66%, 24%, and 6%, respectively, whereas in healthy controls the frequencies of CC, CT genotypes we CC, CT, and TT genotypes re 62%, 30% and 8% respectively. No significant association was found between PTPN22 C/T SNP and patients with GD. Conclusion: GD is not associated with PTPN22 C/T SNP in Kumaon population. Furthermore, C/T SNP in PTPN22 gene could be a part of variation in different ethnic populations across the globe.

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Introduction

The protein tyrosine phosphatase non-receptor type 22 (PTPN22) locus has a strong and consistent genetic association with autoimmune diseases. This phosphatase is expressed in hematopoietic cells and in immune cells with highest levels found in neutrophils and natural killer cells. [1] The PTPN22 gene is located on chromosome 1p 13.3–13.1 and encodes the cytoplasmic lymphoid specific phosphatase (Lyp). [2] Single Nucleotide Polymorphisms (SNPs) have been identified in PTPN22, but only one non-synonymous SNP has been intensively in relation to autoimmune diseases. The SNP is a change of cytosine to thymidine at nucleotide which results in an amino acid change from arginine to tryptophan at codon 620 (R620W). This codon is located in the polyproline binding motif P1.[3] The amino acid substitution is located in the polyproline motif within the Lyp protein and, thus, is thought to be involved in binding to SH3 domains during protein–protein interactions.[4] Lyp interacts with C-terminal Src kinase (Csk) to regulate both B-cell receptor (BCR) and T-cell receptor (TCR) signaling. [5,6] The C/T polymorphism increases Lyp protein activity resulting in inhibition of T-cell signaling and a failure to delete autoreactive T-cells during thymic selection.

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The C/T polymorphism results in immune responses against autoantigens genetic association is proposed to be restricted to disorders that have a strong autoantibody component. [7] There is no consensus whether polymorphism is a gain-or loss-of-function variant. The susceptibility locus associated with several autoimmune diseases, it was first reported in type 1 diabetes mellitus (T1DM). [8] The implication of the PTPN22 polymorphism was also proposed in other autoimmune diseases.[9] The implicated in the genetic basis of autoimmunity, the polymorphism may protect individuals from environmental pathogens. [10] The advent of new genotyping and other molecular biology technologies has provided a huge increase in the quantities of data available for analysis. In epidemiology, candidate-gene and genome-wide association identified a large number of genes associated with diseases.[11]Meta-analysis has been widely used as a powerful approach to identify true-positive associated genes, but several limitations can alter the results.[12] Clustering of diseases within families may be explained by shared environmental exposures, shared genes, or interactions between genetic and environmental factors.[13] PTPN22 is specifically expressed in lymphocytes.[14] The through formation of a complex with C-terminal Src Kinase (CSK) suppresses the downstream mediators of T-cell receptor signaling.[15] A growing body of evidence suggests that exposure to cigarette smoke, for example, may increase the risk of multiple autoimmune diseases, including AITD.[16] PTPN22 is associated with several different autoimmune disorders, PTPN22 as the first major genetic variant that clearly confers substantial risk of multiple different autoimmune phenotypes, and it points the way to understanding common disease mechanisms.[17] This study also investigated the association between gene polymorphism SNP study between patients and control group compare and related to the development of GD in Kumaon population.

Material and Methods

Study subjects

A case control study was carried out on fifty individuals, divided into fifty GD patients and fifty healthy control group.

Controls group

The control group contained fifty (seventeen male and thirty-three female), apparently healthy individuals. They were selected randomly from relatives of patients and other volunteers. They were free from symptoms and signs of family history of thyroid disease and no goiter on examination; they had normal thyroid functions and were negative for thyroid autoantibodies and no any chronic diseases such as DM, cardiac diseases, heart diseases, hypertension, renal diseases or others. All cases completed detailed included the essential information, i.e., age, sex, family history, medicine history, and any other relevant information.

Patient group

It contained fifty patients with GD (Eleven male and thirty-nine female), Patients were selected and diagnosed from by specialist doctor team in tertiary care referral hospital, ENT Out Patient Department in Dr. Susheela Tiwari Government Hospital, Haldwani, Nainital, Uttarakhand. The GD was diagnosed by: (1) documented clinical and biochemical hyperthyroidism requiring treatment, (2) a diffuse goiter, (3) presence of TSH-receptor antibodies. For all subjects, phenotype was determined with the clinician blinded to the individual's genotype.

Collection of Blood Samples

Blood samples were collected, from the ante median cubital vein of the subjects using disposable plastic syringes with all aseptic precautions. Blood was transferred immediately in to a dry clean plastic test tube with a gentle push to avoid hemolysis. Blood was collected in EDTA vial (Levram Lifesciences Silvassa, India) from both groups (Healthy Control and T2DM patients) for molecular research studies. The research was done in the Multidisciplinary Research Unit (DHR-ICMR, New Delhi), Government Medical College, Haldwani, Nainital, Uttarakhand, India.

Genomic DNA Extraction

Genomic DNA was isolated from human blood samples by using genomic DNA extraction kit (GeneJET Genomic DNA Purification Kit, Thermo Fisher Scientific, USA) as per the manufacturer's instructions using centrifuged (Eppendorf 5424R, Germany). After extraction, DNA samples (working) was stored at 4°C for 7 days before spectrophotometric (analysis and then stored in a freezer at −20°C (Vestfrost, Denmark). DNA concentration and purity were measured by ultraviolet (UV) spectrophotometry using an Eppendorf Biospectrophotometer (Eppendorf, Hamburg, Germany) using 1 µL of each sample. The spectra were recorded wavelength range of 220–830 nm.

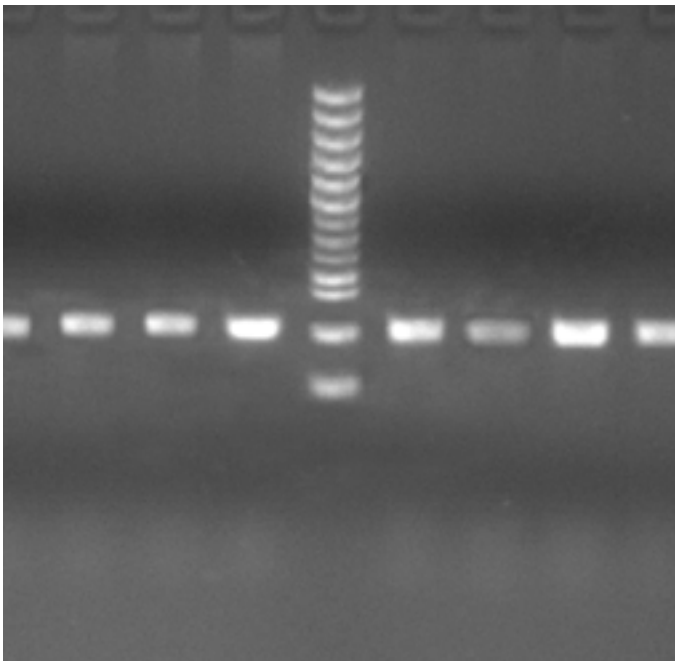
DNA Integrity and Agarose gel electrophoresis

DNA was analyzed by agarose gel electrophoresis (Bio-Rad Mini Gel Electrophoresis Unit, USA) using 0.8% agarose gel (Ameresco USA). Electrophoresis was performed using 10X TBE Buffer (Tris-borate-EDTA) (Thermo Scientific, USA) buffer containing 1 µg/ml of Ethidium Bromide (EtBr) (VWR Amresco Life Science, USA) and a constant voltage of 100 V for 50 min using PowerPac Universal (Bio-Rad Laboratories, USA). The DNA bands were visualized and images were acquired using Gel Doc XR+ Imaging system (Bio-Rad Laboratories, USA).

Polymerase Chain Reaction study of PTPN22 gene polymorphism

Oligonucleotide primers were synthesized (Eurofins Genomics India Pvt. Ltd., Kerala, India), PTPN22 gene polymorphism. Polymerase Chain Reaction (PCR) to amplify the polymorphic regions by primer of Forward, 5' TCACCAGCTTCCTCAACCACA 3' Reverse, 5' GATAATGTTGCTTCAACGGAATTT 3' size of PCR product 218 bp.[18] The primers for the PCR were as follow by first PCR master mixture was prepared. Reactions were performed in a 25 µl volume containing 12.5 µl of the DreamTaq PCR master mix (2x) Thermo Fisher Scientific, USA (containing DreamTaq DNA polymerase, 2X DreamTaq buffer, 0.4 mM of each dNTP and 4 mM of MgCl2), 0.5 µl each of 10 ng/µl forward and reverse primers (Eurofins Genomics India Pvt Ltd, Kerala, India), 11 µl of nuclease free water (Thermo Fisher Scientific, USA) and 0.5 µl of positive controls or nuclease free water for no template controls (NTC) per 25 µl of reaction mix in 0.2 ml flat cap PCR tubes (Axygen Scientific, USA). The mixture was overlaid with mineral oil and subjected to PCR amplification and PCR conditions for amplification of a 218 bp fragment of PTPN22 gene polymorphism (Fig.1), 35 cycles of PCR consisting of denaturing for 30 sec at 95°C, annealing for 30 sec at 60°C, extension for 1 min at 72°C and a final extension for 10 min at 72°C using the program temp control Thermal cycler System (Applied Biosystems ProFlex PCR System, USA).

Figure 1: Agarose gel electrophoresis (3% agarose gel) showing fragment of 218 base pair PCR product detect in PTPN 20 gene Lane M: 100-bp DNA ladder; Lane 1 to Lane 14: 218 bp PCR product



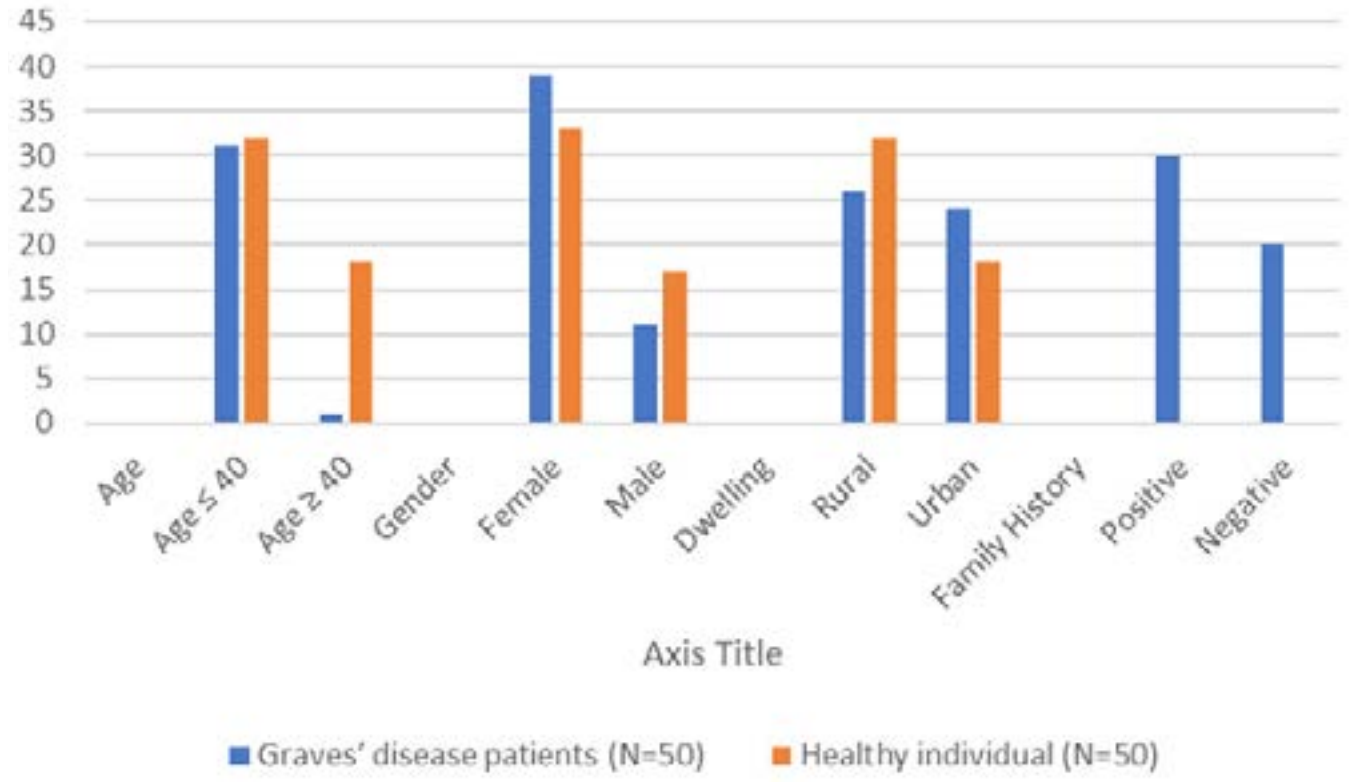
XcmI digests the PCR product, one band of 218 bp is not digested with CC homozygote, two band of 176 bp and 44 bp digested with CC homozygous and three of 218 bp, 176 bp and 44 bp with heterozygous C/T. The digested PCR products were electrophoresed on 3% agarose gels to separate the fragments.The digested PCR products were electrophoresed on 3% agarose gels to separate the fragments. The laboratory data were expressed as means ± standard deviation (SD). Statistical analysis was performed using the Statistical Package for the Social Science program (SPSS for Windows, latest Version 10.3). In this study, a two-tailed P-value less than 0.05 was considered significant.

Results

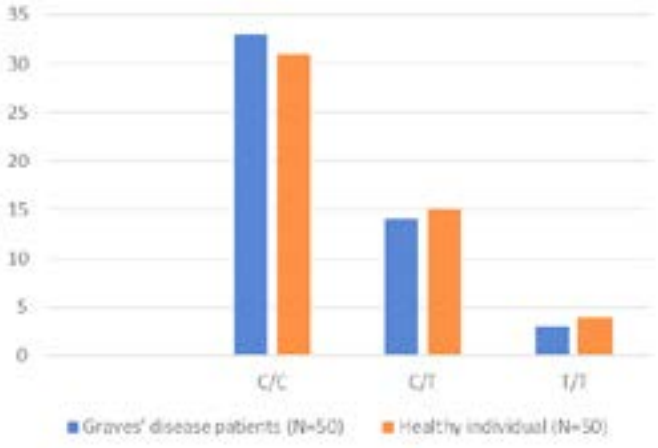
A summary of selected characteristics, including age, gender, thyroid size and family history of graves' disease patients (n=50), and healthy individual (n=50) are presented in (Table I). The frequency matching variables were compared between the cases and controls. All individuals belonged to the Kumaon population. Maximum number of GD patients in age group below then 40 years (62%) as compare above then 40 years (38%). Matching of gender was imperfect, the cases had a markedly higher percentage of female (78 %) than the controls (66%), due to GD predominantly affecting females and in GD patient's male (22%) than the control (34%). In our study graves' disease patients' residence in rural area (52%) and in urban area (48%). Maximum number of patients residence in rural area due to awareness and knowledge. Those patients' residence in rural area they were in nearby industry and industrial area because environmental exposures confer an increased risk of autoimmune thyroid disease (Bar Chart:1).

Family history of graves' disease patients in this study (60%) and without family history was (40%) graves' disease patients. The genotype frequencies of graves' disease patients PTPN22 were 66% (CC), 28% (CT), and 6% (TT) and genotype frequencies of healthy individual 62% (CC), 30% (CT) and 8% (TT). The genotype frequencies of graves' disease patients the parameter of odds ratios (ORs) and 95% confidence intervals (95% CIs) was 4.23 (0.87-20.62) and the p value < 0.05. The genotype frequencies of graves' disease patients of Allele T (18%) and Allele C (82%) and genotype frequencies of healthy individual Allele T (28%) and Allele C (72%). The detailed genotype and allele frequency distribution of PTPN22 gene polymorphism were shown in (Bar Chart 2).

Bar Chart:1 Demographic characteristics of all the participants in this study



Bar Chart 2: Genotypic and allele frequency of PTPN 22 C/T polymorphism patients with Graves' disease patients and healthy individual



Discussion

PTPN22 is located at chromosome 1p13.2 and encodes a tyrosine phosphatase that is expressed by hematopoietic cells and acts as a key regulator of immune homeostasis through inhibition of T-cell receptor signaling and promotion of type I interferon responses.[19] PTPN22 has been identified as the main susceptible gene for multiple autoimmune diseases.
[20] This gene is considered the second-most important predisposing gene for human autoimmune diseases, after HLA. Therefore, abnormalities of PTPN22 can lead to the occurrence of autoimmune diseases. The PTPN22 gene encodes lytic tyrosine phosphatase (LYP), which comprises 807 amino acid residues.[21] LYP acts as an inhibitor of T cell activation by binding to variety of signal transduction molecules, such as Csk kinase, which is active in T cell activation. Arginine substitution for tryptophan at codon 620 of the LYP protein (R620W) has been associated with increased risks of rheumatoid arthritis and systemic lupus erythematosus (SLE). [22] The PTPN22 R620W SNP elicits a functional change in LYP, such that the tryptophan-bearing LYP allele cannot bind the C-terminal src kinase (Csk) [23]; this causes proliferation of T cells. Concomitantly, the levels of several Ig isotypes are increased.[24] Among these antibodies, levels of IgG and IgG4 were positively correlated with the titre of anti-thyroperoxidase antibody (anti-TPO).[25] Changes in level of anti-TPO correlated positively with the development of hypothyroidism and an increased inflammatory reaction.[26] We hypothesized that the mechanism of the missense mutation in PTPN22 (N26S) is similar to that of R620W SNP. LYP is an approximately 105-kDa Class I protein tyrosine phosphatase (PTP) which includes a C-terminal PEST-enriched domain and a classical N-terminal protein tyrosine phosphatase catalytic domain; these are separated by an approximately 300-amino acid interdomain. [27, 28] The enzyme includes
four putative polyproline motifs (P1-P4). [29] The missense mutation PTPN22 (N26S) is located on the classical catalytic domain of the N-terminal protein tyrosine phosphatase; however, little is known regarding its specific function. The inter domain of LYP harbours protein-protein interaction motifs and putative phosphorylation sites. The direct intramolecular interaction and inhibition of the catalytic domain, the interdomain plays an important role in regulating catalytic activity of the protein.[30] On the basis of a constitutive interaction between the N-terminal SH2 domain and the catalytic domain, SHP- 1 is inhibited; it is released following recruitment of the domain to phosphorylated targets. [31] Changes in the interactions between domains can indirectly mediate the functional effects of protein interactions and post-translational modifications located in other parts of the protein. The R620W SNP affects interactions among domains in LYP, leading to the occurrence of HT. Identification of the specific molecular mechanism requires further research. The patients with HT were all females in this family, consistent with the previously reported finding that the incidence of thyroid disease in females is much higher than in males.[32] preponderance of thyroid autoimmunity in females is most likely due to the influence of sex steroids. Furthermore, the presence of TPO autoantibodies is the strongest risk factor for both hyperand hypothyroidism; smoking is negatively correlated with the presence of TPO antibodies. Overall, the incidence of HT in females was significantly higher than the incidence of HT in males.[33] The missense mutation of PTPN22 (A77G), but did not exhibit any thyroid disorder. Men are less susceptible to autoimmune disease than women, which may be why III-3 was unaffected. Autoimmune diseases occur most frequently between the ages of 45 and 65.[34] The PTPN22 may be similar to the R620W SNP as a risk locus, which can increase the risk of thyroid disease; however, it may not show disease in carriers.

Conclusion and Future Directions

The application of molecular biology to the study of AITD has undoubtedly made the significant progress in determining the complex factors that lead to the development of AITD. Another important aspect of these findings is the analysis of the functional consequences of AITD susceptibility genes variants and the search for genotype-phenotype correlations. It is clear that in the near future new susceptibility genes for AITD and the mechanisms through which they contribute to the disease development will be identified. This will enable the rapid identification of those individuals, who are at higher risk for AITD before the clinical symptoms. Hopefully, these new discoveries will also be reflected in improved therapeutic targets and novel treatments in the near future. Treatment options will be presumably directed to more preventive strategies, as opposed to the palliative methods of the present management. The therapy of patients with clinical symptoms of the disease will be personalized by targeting the specific pathways that lead to the development an individual's subtype of AITD. Gene therapy is a promising treatment option for a number of diseases and is gaining more and more importance regarding treatment of autoimmune disorders. In AITD vital interaction has been documented between thyroid specific genes, susceptibility genes, environmental factors and immunological synapse genes. It has been proven that these relationships are involved in the genetic susceptibility to AITD and are helpful in the differentiation of AITD phenotypes and response to the therapy. Epigenetic mechanisms of interaction between environmental (IFNα) and genetic (TG) factors trigger the AITD. Future studies on genetic and epigenetic variations will make it possible to quantify the precise effect of specific susceptibility genes and/or epigenetic variation in estimating the heritability. The relationship between susceptibility genes, environmental factors and epigenetic modulation results in breakdown the self-tolerance leading to AITD.

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Conflict of Interest

None of the authors of this paper have a financial or personal relationship with other people or organization that could inappropriately influence or bias the content of the paper.

Authors' contribution

SKS completed the molecular biology research work and approved final version of manuscript. SG provided clinical guideline of this study and prepared draft of manuscript. VS helps in interpretation, screening and clinical data collection of samples of this study.

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