Genetic Screening for SLE: A Validated qPCR Assay for PTPN22 SNP Polymorphisms

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Abstract
Objective: Systemic Lupus Erythematosus (SLE) is a complex autoimmune disease. Multiple factors can trigger this dysregulation of the immune system, including genetic factors such as mutations in the PTPN22 gene. The objective of our study is to develop and validate a quantitative polymerase chain reaction (qPCR) assay for detecting polymorphisms in the PTPN22 gene.
Methodology: Using the PTPN22 gene and SNP rs2476601 sequence, two sets of primers were designed. The first set is for sequencing the partial gene PTPN22, and the second set is for SNP detection using qRT-PCR, focusing on the wild-type allele and two key single nucleotide polymorphisms (SNPs), SNP-G and SNP-T. The qPCR assay was designed with allele-specific probes and primers, and with RNaseP used as an endogenous control. DNA from patient and control populations was used to validate the specificity, sensitivity and reliability of the assay across various parameters.
Results: qPCR assay could discriminate wild-type from mutant alleles with good sensitivity and specificity. In SLE patient samples, the predominant mutation identified was SNP-G while the lowest level was that of SNP-T.
Scope: The developed qPCR assay has the ability to measure polymorphisms in SLE and, therefore this development opens up new vistas to investigate genetic variations conferring susceptibility to SLE as well as complications. A validated qPCR assay described here is an efficient method for studying polymorphisms of PTPN22 with a wide range of applications in genetics research and clinical diagnostics with personalized approaches.

INTRODUCTION:

Systemic lupus erythematosus is a complex chronic autoimmune disease that mainly affects young women but accounts for a large proportion of women of African, Asian, and Hispanic origin (1). The disease results in dysregulation of the immune system, thus giving rise to autoantibodies that attack the internal tissues and organs of the body. SLE can present with a wide range of clinical features, such as rashes on the skin, joint pains, kidney dysfunction, and neurological problems, the multi-organ involvement and fluctuating disease activity are a characteristic of SLE. The complete aetiology of this disease is not completely understood; however, recent research suggests that both genetic predispositions and environmental factors contribute to its pathogenesis (2,3).

Due to these heterogeneous presentations, overlapping symptoms with other autoimmune disorders, and the absence of a single gold-standard test makes the diagnosis of SLE a challenge. Current diagnostic criteria rely on a combination of clinical manifestations and immunological biomarkers (4,5). According to recent reports, the global incidence and prevalence of SLE are increasing, and some researchers have put susceptibility to the disease under the genetic polymorphisms, especially PTPN22 gene (6,7). Since the biomarker-based diagnosis poses limitations, there is a growing interest in exploring the genetic factors that are related to the incidence of SLE and developing new tools (8).

Studies have explored the PTPN22 gene involvement in autoimmune diseases and found that individuals possessing certain genetic factors are at risk of not only developing SLE but also for developing more severe forms of the disease (9,10). The PTPN22 gene is located on chromosome 1 (chromosome 1p13) and is mainly expressed in immune cells, such as T and B lymphocytes, it encodes a protein in the tyrosine phosphatase

(PTP) family, and these proteins are crucial regulators of the signal transduction process that transmits external signals to the nucleus in cells. The PTPN22 protein regulates T-cell activities, which is the most important cell for the recognition and protection from pathogens and infectious agents. These proteins act as a negative regulator of T-cell receptor (TCR) signalling, which is one of the most important mechanisms for maintaining immune balance and avoiding the activation of excessive immune responses (11,12,13). The LYP protein, which is encoded by the PTPN22 gene, acts to dephosphorylate certain signalling molecules within the pathway of T-cell receptors that are central in maintaining the immune tolerance and arresting processes that could spur a misdirected autoimmune response whereby immune cells attack the very tissues of the body they are meant to protect (14,15,16,17). Being part of both the T-cell and B-cell signalling pathways, PTPN22 must be a part of the process where the immune system distinguishes between self and non-self antigens. Thus, PTPN22 is implied in immune homeostasis and in the prevention of autoimmune diseases (18).

Studies among different populations of diverse ethnicities have provided evidence for an essential interaction of PTPN22 gene polymorphisms and SLE, the 620W variant of the PTPN22 gene was specifically associated with increased risk (13,14). The rs2476601 is one of the SNPs in the PTPN22 gene reported in SLE patients, in this variant the adenine was replaced by guanine (A1858G/T) and with an amino acid change from arginine (R) to tryptophan (W) at the position 620 of the protein (19). This genetic variation results in a gain-of-function mutation within the LYP protein through increased phosphatase activity. Therefore, the signalling of T-cell receptor (TCR) will be downregulated and possibly affect the immune responses that lead to the development of SLE or other autoimmune diseases (20).
Our current study is poised to explore this genetic polymorphism by developing a protocol by using bioinformatics tools to identify PTPN22 gene polymorphism and creating a base for the development of a quantitative PCR (qPCR) assay that detects PTPN22 polymorphisms associated with systemic lupus erythematosus (SLE). The protocol encompasses an integrated approach to be followed, which includes SNP determination, primer and probe synthesis, and optimization of reaction conditions. In addition, the integration of biochemistry with bioinformatics has been tried and successfully achieved, thus paving the way for developing novel genetic tests to detect polymorphisms.
The present study will therefore provide a framework for researchers to diversify qPCR assays for SNP detection. Moreover, it provides an indication of future prospects in these assays regarding personalized treatment strategies that may eventually lead to the identification of the genetic basis of diseases and targeted therapeutic interventions.

MATERIAL AND METHODS:

This study discusses the development of a practical protocol for the development of a qPCR assay detecting PTPN22 gene polymorphisms in SLE.

1. Primer and Probe design

Designing primers for qPCR on SNP (Single Nucleotide Polymorphism) assay requires careful consideration of the target SNP, design principles of the primer and requirements of the assay.
Two sets of primers need to be designed as per the sequence of the PTPN22 gene and SNP rs2476601. The first set will be for sequencing the partial gene PTPN22 while the second set will be for detecting SNP using qRT-PCR. Primer3 and Primer-BLAST can be used for retrieving the sequences and designing the primers.

2. Sequence retrieval and identification of SNP location

Retrieve the sequence surrounding rs2476601 from NCBI (or Ensembl) using the PTPN22 gene ID. The SNP sequence for primers and probes design used here is provided below. The SNP rs2476601 was identified as a potential genetic marker in SLE susceptibility according to the meta-analysis conducted by de Lima et al (2017) (21).

>rs2476601[Homosapiens]
ATTAAAGGCATGAGCCACCATGCCCATCCCACACTTTATTTTATACTTACTGA
ACTGTACTCACCAGCTTCCTCAACCACAATAAATGATTCAGGTGTCC
[A/G/T]
TACAGGAAGTGGAGGGGGGATTTCATCATCTATCCTTGGAGCAGTTGCTATC
CAAAATGTCAAAAATATTGTAACAATTGTTAATTAGAACAATCCAAAG

3. Primer and Probe Design Criteria

- Amplicon size: 70-150 bp (Optimal for qPCR
- Primer length: 18-25 nucleotides long to ensure specificity
- GC content: 40-60% to facilitate efficient primer binding

Avoiding self-complementarity: It should be ensured that primers do not have any self-complementarity, as it could lead to primer-dimer formation where the 3′ end of the primer doesn't pair with another primer. There are certain hairpin loops also that can fold back on themselves, creating hairpin structures. Primer3 tool was used to check this, which provides a detailed report on primer-dimer and self-complementarity.

Avoiding cross-reactivity: Cross-reactivity can occur when primers bind to unintended genomic regions, leading to non-specific amplification. Checking whether the primers bind to multiple regions in the genome by running them through BLAST search will ensure that, primers are highly specific to the target sequence. The repetitive elements like Alu sequences may also bind non-specifically (Dunbar, S. A 2014). Tools like Primer-BLAST (NCBI), In-Silico PCR (UCSC) and GenomeTester was used for this.

Avoiding regions prone to mutations: Primers should be designed in regions that are less likely to have genetic variations, such as SNPs (single nucleotide polymorphisms) or indels (insertions/deletions), as these can affect binding efficiency and result in amplification failure or biased results (23). Some of the simple approaches we can employ here are;

a) To check SNP databases like dbSNP to identify common polymorphisms in our primer regions.
b) Target Exonic/Intron-Exon Boundaries carefully: Primers should avoid areas with frequent indels or splice site variations.

These steps were completed using tools like dbSNP (NCBI), Ensembl Genome Browser and Primer3Plus (24).

Melting Temperature (Tm): The melting temperature should be within a suitable range to promote efficient annealing during PCR, and the optimum conditions are 58- 60°C for primers and typically ~70°C for probes.

SNP Detection: For SNP detection we can use allele-specific TaqMan probes. A TaqMan probe contains both a fluorescent reporter dye (such as FAM: Fluorescein amidites) attached to the 5′ end and a quencher dye (such as TAMRA (5-Carboxytetramethylrhodamine) or BHQ (Black Hole Quencher)) attached to the 3′ end. The probe is designed to bind specifically to the SNP region. The fluorophore emits a signal when the probe binds to the target sequence, and this signal is detected during the qPCR.

Practical considerations: For allelic discrimination, two probes are typically designed: one for the wild-type allele and one for the mutant allele. Use FAM for one allele and VIC for the other to distinguish between them based on fluorescence. We also need an appropriate quencher like TAMRA or a non-fluorescent quencher like BHQ (Black Hole Quencher) (25).

4. Ordering Instructions for primers and probes

After the successful completion of validation of primers and probes designed, we can order the developed sequence (See Table 1: Primers used for sequencing the PTPN22 gene, Table 2: Primers used for qRT-PCR for SNP detection). For the current study Primers and probes were ordered through BioDeskINDIA Labs. (Synthesis scale: 25 nmol to 50 nmol).

5. Validation

Primer performance should be validated by experimental testing of the designed primers using PCR to verify their specificity and efficiency in amplifying the target DNA region, with appropriate positive and negative controls (26).

6. Gene identification by PCR

6.1. PCR Standardization

PCR standardization involves optimizing the PCR conditions to ensure consistent and reliable amplification of the target DNA using the designed primers (27). The steps for PCR standardization involve

- **Primer validation:** Before starting the PCR standardization, the designed primers should be validated by performing PCR with the intended template DNA and checking for specific amplification of the target region. Appropriate positive and negative controls should be used here.
- **Template DNA preparation:** The template DNA to be used in the PCR standardization could include purified genomic DNA, plasmid DNA containing the target sequence, or synthesized DNA fragments.
- **Optimization of PCR components:**

DNA template: The optimal amount of template DNA to use in the PCR reaction should be determined and template DNA concentration titrated to find the optimal range that provides a strong, specific amplification signal without inhibition or nonspecific amplification.

- Primers: Primer concentrations also should be optimized to achieve efficient amplification. Different primer concentrations and primer gradients should be tested to find the optimum concentration that give robust amplification.
- Mg2+ concentration: Magnesium ions, Mg2+, are important cofactors in DNA polymerase enzyme activity. Therefore, variation of the Mg2+ concentration in PCR might reflect changes in efficiency; try varying concentrations of magnesium ions, Mg2+.
- dNTPs concentration: The concentration of deoxynucleotide triphosphates (dNTPs) optimized to ensure sufficient concentrations for DNA synthesis during PCR.
- PCR cycling parameters:

a) Annealing temperature: A few annealing temperatures should be tried to find out the best one that caused specific primer annealing without any nonspecific binding. Gradient PCR or testing of different temperatures individually determined the optimum annealing temperature.
b) Extension time: This should be optimized for sufficient DNA amplification so that nonspecific amplification or primer-dimer does not occur. Different extension times were screened to identify the correct time.
c) Denaturation and first activation: The time and temperature of the denaturation and first activation step of the PCR reaction are very critical. It is determined by the DNA polymerase used.

6.2 PCR reaction setup:

- Preparing master mix: The volumes of PCR component for multiple reactions were calculated and a master mix was prepared to ensure similar reactions across all.
- It contained the appropriate positive and negative controls to monitor the specificity and contamination of the reaction, respectively. It included a positive control with known target DNA and negative controls without template DNA.
- Amplification and analysis: Standard PCR reactions were performed. The PCR products were confirmed to specifically amplify the targeted region through gel electrophoresis and sequencing. Optimization refinement: The PCR conditions were further refined by adjusting specific parameters based on the results obtained during the standardization process. The optimization steps should be repeated until optimal and consistent amplification is achieved (28).

6.3 Testing of primers and probes

Primers were validated by performing PCR with the intended template DNA and checking for specific amplification of the target region using appropriate positive and negative controls. Conventional resin-based DNA extraction or Magnetic bead-based DNA extraction can be employed for gene extraction and to check the purity and PCR yield, agarose gel electrophoresis and UV-spectrophotometry can be done.

Table 1: Primers used for sequencing the PTPN22 gene

	Sequence	Length	Tm	GC%
Forward(SLELGF)	GAAAGTGCTGGGATTAC	17	54	47.1
Reverse(SLELGR)	GCTGAGGATAAGGATTTG	18	55	44.4

Table 2: Primers used for qRT-PCR for SNP detection

	Sequence	Length	Tm	GC%
Forward	CCAGCTTCCTCAACCACAATA	21	62	47.6
Reverse	TGGATAGCAACTGCTCCAAG	20	62	50
Probe	FAM-	24	67	58

A(wildgene)	CCCTCCACTTCCTGTATGGACACC-BHQ1			
Probe G(SNP-Gvariant)	FAM-TCAGGTGTCCGTACAGGAAGTGGA-BHQ1	24	68	54
Probe T(SNP-T-variant)	FAM-AGGTGTCCTTACAGGAAGTGGAGGG-BHQ1	25	68	56

*Human Ribonuclease P was used as the housekeeping gene for the study.

Table 3: House-keeping gene primer used.

	Sequence
Forward	AGATTTGGACCTGCGAGCG
Reverse	GAGCGGCTGTCTCCACAAGT
Probe	FAM-TTCTGACCTGAAGGCTCTGCGCG-BHQ1

All the gene-specific primers and housekeeping gene primers were validated by PCR using the DNA from the 5 known SLE samples and 5 healthy controls.

Step 1> DNA extraction
DNA was extracted from EDTA blood samples of 5 known SLE samples and 5 healthy controls using DNA Extraction Kit and the DNA quantity and quality analysis were done using UV-spectrometry and agarose gel electrophoresis.

Step 2> PCR standardization, Validation and Gene Sequencing
Using the SLE LGF and SLE LGR primers, the partial area of the PTPN22 gene was sequenced which includes the SNP region with an amplicon size of 878bps.

Sample collection for validation of the developed Assay

- A total of 10 blood samples were collected from SLE patients and healthy individuals and the collected samples were used for DNA extraction and stored for further use in validation procedures.
- Healthy samples are labelled HS1 to HS5 and Patient samples are labelled as PS1 to PS5.

RESULT

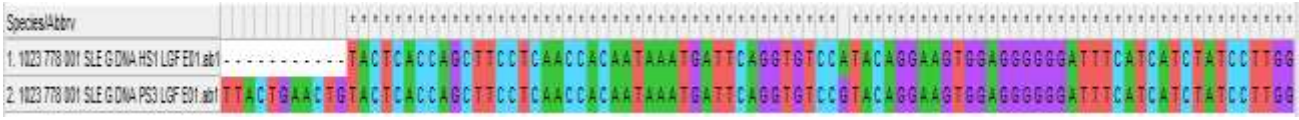


Figure 3. Multiple sequencing alignment of sequencing result (SNP region is labelled by using an arrow.)

DNA was extracted from the 5 SLE patients' samples and from 5 healthy control blood samples, quantified by Spectrophotometry and quality was checked by Agarose Gel Electrophoresis (AGE) then the extracted genome underwent the designed qPCR Assay for validation.

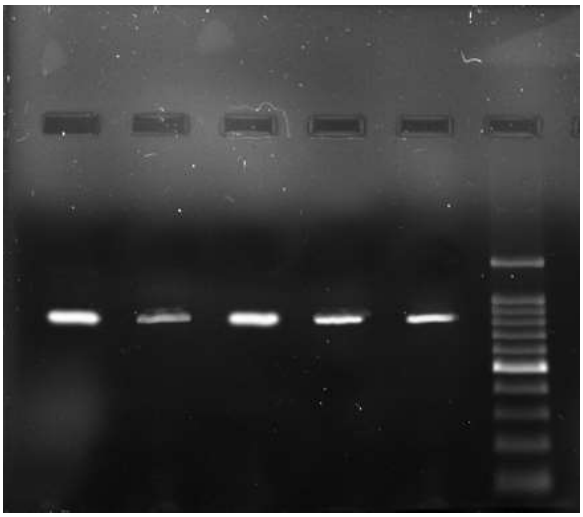


Figure 1. AGE image showing PTPN22 gene partial amplification for healthy samples

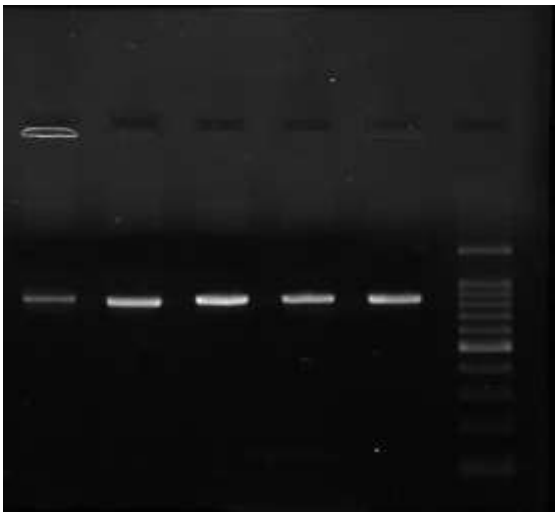
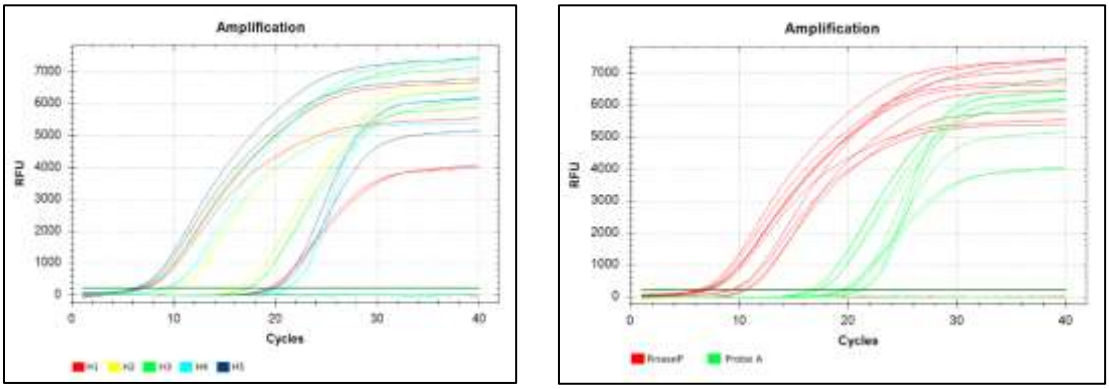
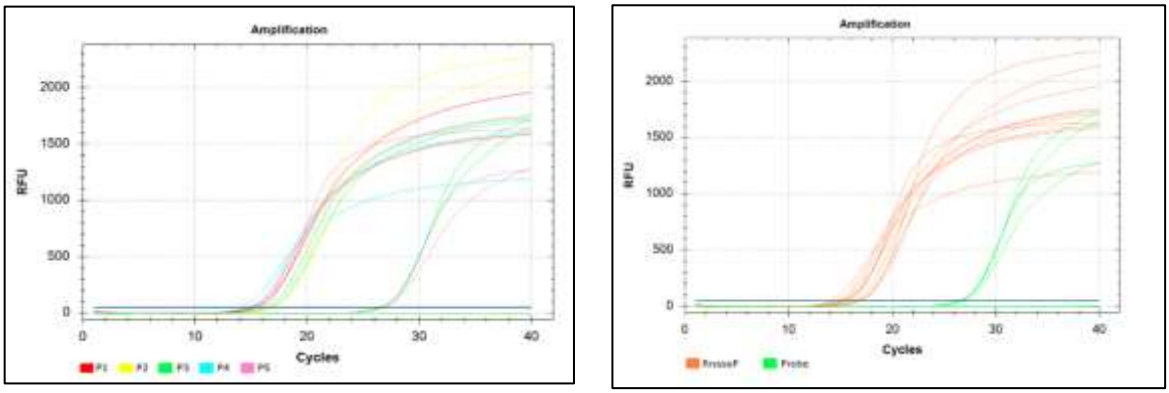


Figure 2. AGE image showing PTPN22 gene partial amplification for patient samples.

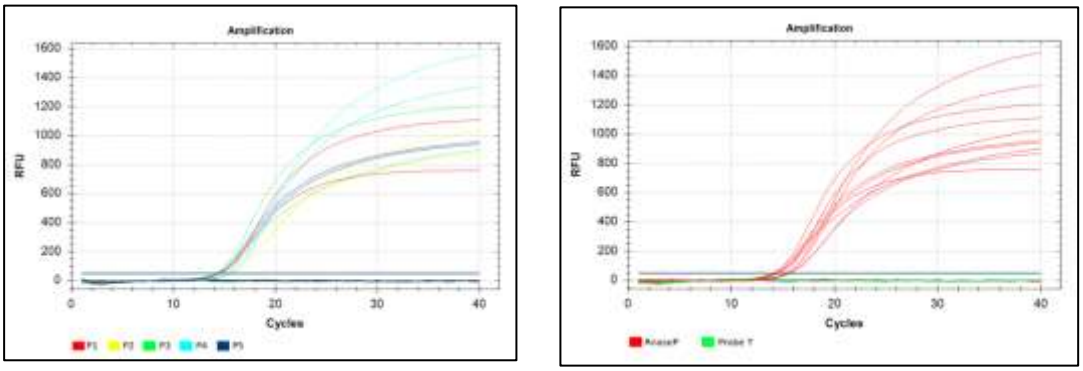
Both Figures 1 & 2 of AGE show 10 samples of the PTPN22 gene have been amplified and *are* in good quality, there were sharp bands observed and no additional bands or smearing.



Graphs 1 and 2: Amplification of probe A in Healthy samples. Amplification curves for Probe A, which detects the wild-type version of the PTPN22 gene, and RNaseP (used as a control) for healthy individuals (H1 to H5). The red curves represent the consistent amplification of RNaseP, confirming that the DNA samples were of good quality and that the qPCR test worked properly. The green curves show the detection of the wild-type allele (A) in all the healthy individuals. The early amplification (low Ct values) suggests a high concentration of the wild-type allele, which is typical for people with the A/A genotype. There is very slight variation in the results, indicating that mutations (like SNP-G or SNP-T) are unlikely in this group.



Graph 3 and 4: Amplification results for Probe G identifying the PTPN22 gene with the SNP-G mutation and RNaseP, the control, in patient samples P1 to P5. The red curves indicate uniform amplification of RNaseP in all samples and ensure that DNA quality was good, therefore validating the qPCR assay. The coloured curves for Probe G indicate that the patients were carriers of the SNP-G allele. Samples P2 and P3 have an earlier Ct value, meaning it has a higher concentration for the G allele and possibly a homozygous mutant or G/G genotype. Those samples such as P1 and P5 with more delayed Ct values mean lesser concentration of the G allele, indicating heterozygous genotypes such as A/G or G/T. These amplification curves indicate that the SNP-G mutation is present in the SALE patients at different levels and, therefore, reflects the efficiency of the qPCR assay in detecting this genetic variant.



Graphs 5 and 6: Amplification curves of Probe T for the PTPN22 gene's SNP-T mutation and of RNaseP (Internal control) for both patients and healthy individuals. The identical amplification of RNaseP indicates good DNA quality and specificity of qPCR. Amplification curves of Probe T are green and show no or late amplification; thus, the SNP-T allele is absent or at very low levels in most samples. This means that the T mutation is very rare in the patients and healthy individuals, and possibly only heterozygous genotypes A/T or G/T, in a few cases. Overall, it indicates the low prevalence of the SNP-T allele in our samples.

DISCUSSION:

Chen et al. (2021) designed a new qPCR assay targeting specifically the detection of SNPs. This was implemented instead of the traditional procedures that usually are cumbersome and time-consuming, requiring extensive optimization by using a mixture of various primers and probes within a single reaction tube. With the novel design, they substantially shortened the overall development time for the assay while maintaining a high level of accuracy. The qPCR assay is therefore ideally suited for emergency applications or in any other situation where time is a limiting factor. Similarly, the present study was able to successfully create a qPCR assay to detect SNPs; in this study, it was the PTPN22 polymorphism (rs2476601) associated with SLE. The SNP has been instrumental in identifying mechanisms of disease and therefore patient outcomes. SLE is a complex disorder requiring careful diagnosis and treatment strategies. Personalized treatment approaches have the potential to significantly improve the overall management of diseases, and this particular type of qPCR assay not only simplifies the diagnosis process but also facilitates a more effective management strategy for patients suffering from Systemic Lupus Erythematosus (SLE). Furthermore, in the study conducted by

Kyogoku et al., in the year 2004, it was reported that the 'C' allele was identified as the predominant allele associated with the PTPN22 R620W SNP polymorphism, which is denoted as rs2476601 (29).
The 'T' allele (W620), although the minor allele, had a frequency of 12.67% in SLE cases and 8.64% in controls. Similarly, in our study, SNP-G (the complementary strand of the C allele) was identified as the predominant allele in SLE patients, while SNP-T was observed at a lower frequency while validating using the developed qPCR assay with SLE-positive samples and healthy controls. Therefore, the qPCR assay developed in this study proved that this assay can be a reliable and valuable tool for genetic studies of SLE and holds great potential for future applications in clinical diagnostics. Its accuracy in detecting SNPs contributes to the advancement of targeted therapies and personalized treatment strategies, ultimately aimed at improving patient outcomes.

CONCLUSION:

This study developed and validated a high-quality qPCR assay for the detection of PTPN22 polymorphisms, and as per the validation, the assay shows high specificity, and sensitivity and reliably distinguishes between wild-type and mutant alleles in patient populations and control populations. Among the SLE patients, SNP-G was the predominant mutation, and SNP-T occurs at lower frequencies. This qPCR assay would be useful in the genetic study of SLE and may be of significant use in the future in the context of clinical diagnosis and personalized medicine. It is founded upon the ability to detect and quantify these polymorphisms, which makes it a good basis for further research into the mechanisms of genetic causality for SLE and the development of targeted therapies, especially towards devising personalized treatment strategies.

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CONFLICT OF INTEREST

We have no conflict of interest to disclose.

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