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ASSOCIATION OF TLR2 AND IL-8 POLYMORPHISMS AND THEIR EXPRESSION IN GUILLAIN-BARRÉ SYNDROME

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ABSTRACT: Guillain-Barré syndrome (GBS) is an acute inflammatory, autoimmune disorder of peripheral nervous system. Association of TLR2 and IL-8 polymorphism with higher expression level has already been studied in many inflammatory and autoimmune diseases. However, the possible role of TLR2 and IL-8 polymorphism in GBS remains unknown. Therefore, the current study investigated the TLR-2 (Arg677Trp & Arg753Gln) and IL-8 (-251A/T) polymorphisms in 105 GBS patients and 100 healthy controls using polymerase chain reaction-restriction fragment length polymorphism analysis. Further, the expression of TLR-2 and IL-8 gene was determined by western blotting and enzyme-linked immunosorbent assay respectively. TLR2 (Arg677Trp & Arg753Gln) heterozygous genotypes were strongly associated with increased risk of GBS. TLR2 and IL-8 genes showed significantly higher expression in GBS when compared with healthy controls. In conclusion, TLR2 polymorphisms showed significant association with GBS, and their enhanced expressions and increased level of IL-8 have possible role in GBS development. TLR2 polymorphisms could be a genetic marker to GBS susceptibility.

INTRODUCTION: Guillain Barré syndrome (GBS) is an immune-mediated inflammatory disease mainly affecting the myelin and axons of peripheral nerves with heterogeneous pathological features, often triggered by an aberrant immune response towards an infectious pathogen ¹. Epidemiological studies linked it with *Campylobacter jejuni*, Cytomegalovirus, Epstein Barr virus and *Mycoplasma pneumonia* ².

The mechanisms involved in immunopathogenesis of GBS are still unclear. The hypothesis put forward for the immunopathogenesis of GBS points to molecular mimicry between lipopolysaccharide (LPS) and ganglioside like epitopes in host nerve cells, which leads to cross reactivity of immune response after the infection. Besides microbial factors, host susceptibility may also play an important role in the etiology of GBS, because not all infected individuals develop this disorder.

It is estimated that only 1:1000 people develop GBS after *C. jejuni* enteritis, thus highlighting the role of host genetic factors ³. However, limited studies have been conducted for identifying the potential host factors that may impart susceptibility to GBS.

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Toll-like receptors (TLRs) family plays a fundamental role in innate immunity and signal the activation of adaptive immunity⁴. One of the human Toll homologues, TLR2, has been shown to be involved in LPS signalling^{5, 6, 7}. In addition to this, TLR2 is activated primarily by peptidoglycan, spirochetal glycolipids, lipoproteins and lipoarabinomannan^{8, 9, 10}. Activation of TLRs enhances the transcription of several pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α via NF- κ B^{11, 12, 13}. IL-8 chemokine was characterized for its ability in recruitment and activation of neutrophils at inflammatory sites^{14, 15}. IL-8 also promotes inflammatory processes by attracting some subsets of T lymphocytes to the site of inflammation, inducing cytokine production as well as releasing tissue damaging mediators by neutrophils¹⁶.

TLR2 polymorphism at position 753 (Arg753Gln), an exchange of arginine by glutamine was correlated with the incidence of sepsis caused by gram-positive bacteria in human¹⁷. Another polymorphism in TLR2 at position 677 (Arg677Trp) was associated with susceptibility to leprous leprosy¹⁸. Several studies reported that TLR2 polymorphisms predisposed to autoimmune diseases^{19, 20, 21}. So far, only one study had shown association of TLR4 polymorphism with GBS²². Association of IL-8-251A/T polymorphism was studied in autoimmune inflammatory diseases like multiple sclerosis (MS), systemic lupus erythromatosus (SLE) and rheumatoid arthritis (RA)^{23, 24, 25}.

Taking the importance of TLR2 and IL-8 in genetic susceptibility of various diseases, we hypothesized that host factors might determine the intensity of immune response towards microbial ligands, which might play a pivotal role in GBS development. The precise role of polymorphism & expression of TLR2 and IL-8 are yet not understood in GBS. In present study, we therefore investigated the association of TLR2 (Arg677Trp and Arg753Gln) and IL-8 polymorphisms (-251A/T), and their expressions with GBS.

METHODS:

Study population: Patients admitted to Neurology ward, Sanjay Gandhi Postgraduate Institute of

Medical Sciences, Lucknow were enrolled for the study. The Institutional ethics committee granted approval for this study and consent was obtained from all the study subjects. A total of 105 patients (male 80) with GBS, mean age \pm SD, 30.20 \pm 10.98 years, and sex/age matched 100 healthy controls (male 76), mean age 28.12 \pm 16.97 years were included in study. GBS patients were selected on the basis of criteria as described earlier²⁶ and healthy controls were individuals without any history of apparent infectious illness within the preceding 4 weeks. Patients with GBS had not received any immunosuppressive or immunomodulatory treatment in the last 2 months prior to sample collection.

Sample Collection:

Blood samples were collected through peripheral vein puncture during the first 2 weeks after the onset of GBS. Blood in ethylene diamine tetra acetate (EDTA) was stored at -20°C for DNA extraction. Sera from clotted blood were separated and stored at -80°C till further use for enzyme-linked immunosorbent assay (ELISA). Peripheral blood mononuclear cells (PBMCs) were separated from heparinised blood and stored at -20°C for protein extraction.

Genomic DNA isolation:

Genomic DNA was extracted from whole blood using salting out method. DNA samples were stored at -20°C till further use for genotyping.

TLR-2 genotyping (Arg677Trp & Arg753Gln):

Single nucleotide polymorphisms (SNPs) were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. DNA samples of 100 ng/ μ l concentration were used for SNP detection; primer sequences for TLR-2 (Arg677Trp & Arg753Gln) amplifications are shown in table1. PCR amplifications were performed in a 25 μ L volume containing 10 X assay buffer, 200 mM each of dATP, dCTP, dGTP, dTTP, 0.1 mM of each primer, 1.0 U of Taq DNA polymerase (Bangalore Genei, Bengaluru, India). PCR protocols were as follows: initial denaturation for 10 min at 95°C followed by 35 PCR cycles of denaturation for 30 s at 94°C, annealing for 30 s at 58°C, extension for 30 s at 72°C with final extension of 5 min at 72°C. Amplification products

(20 μ l) were digested with 1U of AclI (Fermentas, Burlington, Canada) at 37°C overnight, electrophoresed on 3.5% agarose (Sigma–Aldrich, St Louis, MO, USA) gel, visualized under UV illumination and stained with ethidium bromide.

IL-8-251 A/T genotyping:

IL-8-251A/T genotype was determined by PCR–RFLP as mentioned above. IL-8 specific primers

are shown in **Table 1**. For RFLP analysis, the PCR products (15 μ L) were digested with 1 U of MunI restriction enzyme (Fermentas Life Science, USA) at 37°C overnight, electrophoresed on 3.5% agarose (Sigma–Aldrich, St Louis, MO, USA) gel, visualized under UV illumination and stained with ethidium bromide.

TABLE 1: LIST OF PRIMER SEQUENCES FOR TOLL-LIKE RECEPTOR 2 (TLR2) AND INTERLEUKIN 8 (IL-8) GENE POLYMORPHISMS

| Gene polymorphism | Method | Primer sequence | |
|-------------------|----------|-------------------------|--------------------------|
| | | Forward primer | Reverse primer |
| TLR2 (Arg677Trp) | PCR-RFLP | GGGACTTCATTCCTGGCAAGT | GGCCACTCCAGGTAGGTCTT |
| TLR2 Arg753Gln | PCR-RFLP | GCCTACTGGGTGGAGAACCT | GGCCACTCCAGGTAGGTCTT |
| IL-8(-251T > A) | PCR-RFLP | CATGATAGCATCTGTAATTAAGT | CTCATCTTTTCATTATGTCAG AG |

Western Blotting:

Total protein extracts from PBMCs were prepared by whole cell lysate preparation procedure²⁷ and supernatants were stored at -20°C. Protein concentration of the cell lysate was determined by Bradford assay method²⁸. The constituent proteins of the PBMCs were separated by SDS-PAGE on a 10% separating gel and then transferred to nitrocellulose membranes (Sartorius, Gottingen, Germany). In order to verify the equivalent loadings of proteins in the wells, the gel and the nitrocellulose membrane were stained with Coomassie brilliant blue and Ponceau S respectively (Sigma). Membranes were blocked by incubation in tris-buffered saline (150 mM NaCl, 25 mM Tris pH 7.5), containing 0.05% Tween 20 (Sigma) and 5% non-fat dry milk and incubated overnight at 4°C. Following morning, the membrane was washed in TBS-T (TBS containing 0.05% Tween-20) for 5 min with three changes.

The membrane was then blotted by transferring into a primary anti-rabbit antibody solution (1:1000) and kept at 40°C for 6 hrs. The blotted membrane was washed thrice as described before and then transferred into the secondary antibody solution and probed with anti-goat horseradish peroxidase (HRP) conjugated IgG antibody and kept at room temperature for 1 hr. The membrane was washed thrice with TBS-T. HRP development reagent (ECL detection kit, GE Healthcare, Buckinghamshire, UK) was supplied as two solutions, one containing Lumiglo and the other

containing peroxide. These two solutions were mixed in the ratio of 1:40 and the membrane was immersed in the mixture for 1 min, wrapped with saran wrap exposed to X-ray film and developed. Density of the proteins on the autoradiogram was quantified by Bio-Rad model GS-700 imaging densitometer using molecular analyst software, version 1.5 (Bio-Rad, CA, USA). The autoradiograms were scanned in the transmittance mode at a resolution setting of 150 dpi, using a gray filter. The Intensity of bands were compared on the basis of adjusted volume (mean optical density area in square millimetres). The densitometric scans from three experiments normalized with native β -actin are shown (**Fig.1**).

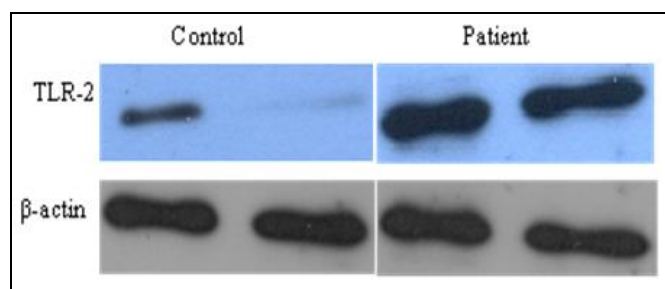


FIG. 1: DENSITOMETRIC SCANS OF REPRESENTATIVE WESTERN BLOTS SHOWING EXPRESSION OF TLR-2 IN CONTROL AND GBS PATIENT FROM THREE EXPERIMENTS, NORMALIZED TO NATIVE β -ACTIN LEVELS

ELISA:

Human IL-8 was measured in the serum using commercial ELISA kit (Invitrogen, Carlsbad, USA) following manufacturer's instruction. All samples were measured in triplicates. For IL-8 serum

samples were diluted to a ratio of 1:100 using assay buffer. The detection limit of the kit for IL-8 is 0.31 ng/ml. The optical density of the wells was determined using a microplate reader set at 450 nm.

Statistical analysis:

The SPSS 16.0 statistical package (Chicago, IL, USA) was used for data management and analysis. Power of the study was calculated using Quanto software version 1.0 (<http://hydra.usc.edu/gxe>) to achieve 80% of the statistical power for Odds Ratio (OR) ≥ 2.0 at significance level (α) < 0.05 . Logistic regression analysis was applied to estimate association with GBS susceptibility after adjusting for age and gender and considered significant if the p values were < 0.05 . Hardy-Weinberg equilibrium was checked in controls by goodness of fit χ^2 test. For comparisons between the groups of study populations χ^2 test was used. ELISA data was expressed as mean \pm SD of triplicate experiments performed independently for each sample. One-way anova: Post Hock (Bonferroni) test was performed to determine the expression level of IL-8 and to compare continuous data (age). The statistical significance of the data of Western blot was determined using Student's t test with Graph Pad Prism software (San Diego, CA, USA) and

triplicate experiments performed independently for each sample.

RESULTS:

Genetic polymorphism:

Genotype and allele frequencies in GBS and healthy controls are shown in **Tables 2** and **3**. Both polymorphisms were in agreement with Hardy-Weinberg equilibrium in controls.

Frequency distribution of the TLR2 Arg753Gln and Arg677Trp Genotype variants in patients with GBS and control groups:

Genotypic distributions of TLR2 Arg753Gln and Arg677Trp polymorphisms demonstrated increased risk of GBS for heterozygous genotypes Arg/Gln ($p < 0.0001$; OR, 8.17; 95% CI, 3.26-20.48) and Arg/Trp ($p < 0.0001$; OR, 86.62; 95% CI, 11.63-644.77). The frequency of wild homozygous genotypes was observed at 65.7% vs 94% for TLR2 Arg753Gln and 53.33% vs 99% for Arg677Trp polymorphisms in patients and controls. Patients with GBS showed significantly higher frequency in comparison to controls for allele 753Gln (17.14% vs 6%, $p < 0.0006$; OR, 3.24; 95% CI, 1.63-6.43) and allele 677Trp (23.34% vs 0.5%, $p < 0.0001$; OR, 60.56; 95% CI, 8.26-443.61) (**Table 2**).

TABLE 2: TOLL-LIKE RECEPTOR 2 (TLR-2) POLYMORPHISMS IN GBS PATIENTS AND HEALTHY CONTROLS.

| Gene polymorphism | Patients (%) | Controls (%) | p value | OR [#] (95% CI) |
|------------------------|--------------|--------------|---------------|--------------------------|
| TLR2Arg753Gln Genotype | | | | |
| Arg/ Arg | 69(65.7%) | 94(94%) | ≤ 0.0001 | 0.12 (0.04-0.30) |
| Arg /Gln | 36(34.3%) | 6(6%) | ≤ 0.0001 | 8.17(3.26-20.48) |
| Gln/Gln | 0(0%) | 0(0%) | -- | -- |
| Allele | | | | |
| Arg | 174(82.86%) | 188(94%) | 0.0006 | 0.30(0.15-0.61) |
| Gln | 36(17.14%) | 12(6%) | 0.0006 | 3.24 (1.63—6.43) |
| TLR2Arg677Trp Genotype | | | | |
| Arg / Arg | 56(53.33%) | 99(99%) | ≤ 0.0001 | 0.01 (0.001-0.08) |
| Arg /Trp | 49(46.67%) | 1(1%) | ≤ 0.0001 | 86.62(11.63-644.77) |
| Trp / Trp | 0(0%) | 0(0%) | -- | -- |
| Allele | | | | |
| Arg | 161(76.66%) | 199(99.5%) | < 0.0001 | 0.01(0.002-0.12) |
| Trp | 49(23.34%) | 1(0.5%) | < 0.0001 | 60.56(8.26-443.61) |

Frequency distribution of the IL-8-251 A/T variant in patients with GBS and healthy controls: To analyze the association of IL-8 polymorphisms with GBS, the genotype frequency was compared between controls and patients with

GBS. The logistic regression analysis revealed no significant association of IL-8-251A/T polymorphism (polymorphic and heterozygous) with GBS ($p = 0.8748$ and $p = 0.0698$) (data is shown in **Table 3**).

TABLE 3: IL-8-251A/T POLYMORPHISMS AND GBS SUSCEPTIBILITY

| Gene polymorphism | Patients (%) | Controls (%) | p value | OR [#] (95% CI) |
|---------------------------|--------------|--------------|---------|--------------------------|
| IL-8(-251A/T) Genotype | | | | |
| A/A | 18 (17.14%) | 29 (29%) | 0.0476 | 0.50 (0.26-0.98) |
| A / T | 60(57.14%) | 44(44%) | 0.0698 | 1.69 (0.97-2.94) |
| T / T | 27(25.71%) | 27(27%) | 0.8748 | 0.93 (0.50-1.74) |
| Allele | | | | |
| A | 96(45.71%) | 102(51%) | 0.322 | 0.80 (0.54-1.19) |
| T | 114(54.28%) | 98(49%) | 0.322 | 1.23 (0.83-1.82) |

Expressions of IL-8 and TLR2:

Enhanced expression of TLR-2 was observed in GBS patients (fold change-1.112) compared to healthy controls (fold change-1) shown in **Fig. 2**. Level of IL-8 was elevated in sera of GBS patients than healthy controls (112.55±13.96 vs 34.79±4.373; $p \leq 0.001$) shown in **Fig.3**.

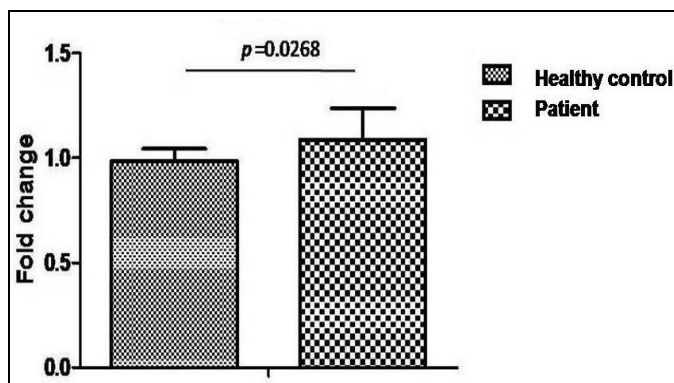


FIG. 2: DATA SHOWS THE ENHANCED EXPRESSION OF TLR-2 IN GBS PATIENTS (FOLD CHANGE-1.112) COMPARED TO CONTROLS (FOLD CHANGE 1).

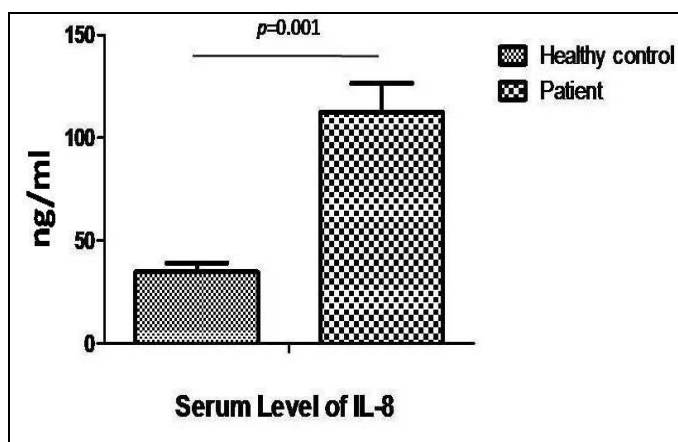


FIG. 3: LEVELS OF IL-8 IN SERUM OF HEALTHY CONTROLS AND GBS PATIENTS

DISCUSSION: In the present study, we investigated the association of TLR2 polymorphism (Arg753Gln and Arg677Trp) with GBS and its relative expression. We also

investigated the expression of IL-8 and its gene polymorphism in patients with GBS. TLRs play central role in host defences and are involved in a number of autoimmune diseases including GBS. The magnitude of the association of polymorphisms with autoimmune disease varies depending on genetics, demographics and environmental factors. Many association studies reported that TLR-2 polymorphisms predisposed to autoimmune disease^{19, 20, 21}. However, some polymorphisms of TLR2 might not be associated with autoimmune disease like rheumatoid arthritis (RA)²⁹. However, there is no data regarding TLR2 polymorphism with GBS till date.

The importance of TLR2 polymorphism in GBS is still largely unknown. In our study, the variant allele frequency of TLR2-753Gln and TLR2-677Trp was higher in GBS than controls (17.14% vs 6%, $p < 0.0006$; OR, 3.24; 95% CI, 1.63-6.43 & 23.34% vs 0.5%, $p < 0.0001$; OR, 60.56; 95% CI, 8.26-443.61). The reported rate of TLR2-Arg753Gln variant frequency was 1% in Spanish population³⁰ whereas the TLR2-Arg677Trp was 30.3% in Croatian population^{30, 31}. In separate studies, the Arg753Gln genotype was observed among 10.34%³² and 12.3%³³ of healthy Turkish subjects, while the Arg677Trp was not observed. Contrary to the study conducted by Kang et al (2001), other authors failed to detect these TLR2 polymorphisms in the Korean population. In the Caucasian population, the TLR2-Arg753Gln SNP was detected in 9.4% of the German whites, while the Arg677Trp polymorphism was not observed at all³⁴.

The data regarding role of TLR2 gene polymorphism in inflammatory demyelinating diseases including GBS is limited. There is a single

study that suggests association of TLR4 Asp299Gly and Thr399Ile polymorphisms with risk for development of GBS²².

We conducted the present study in our population and a positive association of TLR2 polymorphism (Arg753Gln and Arg677Trp) was observed with GBS. Polymorphism in the TLR2 gene causes inappropriate activation of the dendritic cells. These dendritic cells kick off cell maturation and increase the expression of major histocompatibility complex (MHC) and co-stimulatory molecule B7, and activate T cells which secrete increased amount of several chemokines and pro-inflammatory cytokines such as TNF- α responsible for demyelination. There are reports which show that increased level of TNF- α contributes to the pathogenesis of immune mediated demyelinating neuropathies and axonal degeneration by inducing damage to myelin³⁵.

Besides the association of TLR2 with GBS, we also observed the enhanced expression of TLR2 in patient with GBS. The data regarding expression of TLR2 in GBS is very limited. So far, one study showed significantly elevated TLR2 expression in sciatic nerves during GBS³⁶. An increased expression of TLR2 had been shown in synovial tissues of patients with RA³⁷.

In the present study, we found elevated TLR2 expression in PBMCs of patients with GBS (fold change-1.112) when compared to healthy control (fold change 1). TLRs play a central role in the initiation of both innate and adaptive immune responses against microbial pathogens through myeloid differentiation (MyD88) dependent primary response gene or MyD88-independent transduction pathway³⁸. Each member of the TLR family has its own ligand from different pathogens, which helps in inducing a danger signal when pathogen invades the host and results in the activation of NF- κ B and subsequent induction of signal transduction cascade. TLR2 can deliver co-stimulatory T cell signals for cell expansion and can induce proliferation of regulatory T cells³⁹; its signalling favours Th17 cell expansion.

It was shown in the rat model of experimental autoimmune neuritis (EAN) that TLR2 was

expressed in inflamed nervous tissue and NF κ B was increased in activated T cells and macrophages⁴⁰. In EAN, potential endogenous TLR ligands generated following tissue damage or inflammation may also activate their TLRs and thereby play roles in the pathological progress of the disease. TLR2+, CD14+, and Hsp70+ cell accumulation was detected and positively correlated with neurologic disease severity in sciatic nerves of EAN rats, suggesting the involvement of innate immunity in the effect or phase of disease⁴⁰. From our study, we hypothesize that the increased/enhanced level of TLR2 might deliver potent co-stimulatory signals to antigen activated T cells which secrete increased amount of several chemokine and pro-inflammatory cytokines such as TNF- α responsible for demyelination.

Expression of IL-8 and its role in disease pathogenesis in GBS still remain unknown. An earlier study had shown significantly higher IL-8 secretion from PBMCs of MS patients compared to controls⁴¹. In present study, we found the level of IL-8 significantly higher (112.55 ± 13.96 vs 34.79 ± 4.37 ; $p=0.001$) in GBS patients compared to healthy controls. Activation of TLRs enhances the transcription of several pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α via NF- κ B^{11, 12, 13}.

It is also reported that IL-8 and CXCL1 production by human astrocytes at both the RNA and protein levels can be induced by IL-1 β in MS patients⁴². In another study, IL-1 β and IL-17 induced the production of IL-8 in endothelial and parenchymal cell indicating an indirect role in polymorphonuclear neutrophil recruitment⁴³. In another study, the level of IL-1 β was found significantly higher in chicken model of GBS⁴⁴. Elevated expression of TLR2 in GBS as observed in our study indicates that TLR2 activation enhances the transcription of several pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α via NF κ B and IL-1 β in turn stimulates the expression of IL-8 that may have indirect role in recruitment and activation of neutrophil at neuronal sites in GBS.

Besides the IL-8 expression study in GBS, we also looked for the polymorphism of IL-8 gene in GBS but we did not observe the any association of IL-8-251A/T polymorphism with our GBS patients.

Similar observation in another study reported that functional genetic variation in IL-8 did not play a major role in SLE susceptibility in the Spanish population²⁴. In another study, the genetic polymorphisms of the CXCL8 gene were not associated with systemic sclerosis. On contrary to these studies, association of IL-8-251 A/T polymorphism with MS was reported in Iranian patients²³.

In summary, our study indicates that TLR2 polymorphisms and their enhanced expressions were associated with GBS susceptibility in Indian patients. In addition, enhanced IL-8 production without its gene polymorphism was also observed in GBS patients. However, interaction between TLR2 activation and expression of IL-8 in absence of IL-8 gene polymorphisms, and their exact role in disease pathogenesis calls for further study. Similar studies in different ethnic populations are required to clarify the role of TLR2 and IL-8 in GBS patients.

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CONFLICT OF INTEREST: None declared.

AUTHOR CONTRIBUTIONS: NKK and KNP had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept design by KNP and VKP, and DRM; data acquisition, analysis and interpretation by NKK, VKP, MKR and KNP, and manuscript drafting by NKK, DRM and KNP.

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