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ORIGINAL RESEARCH

Toll-Like Receptor (*TLR*) *1*, *2*, and *6* Gene Polymorphisms Support Evidence of Innate Immune Factors in Schizophrenia

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Introduction: Schizophrenia is a complex psychiatric disorder with an important genetic contribution. Immunological abnormalities have been reported in schizophrenia. Toll-like receptor (TLR) genes play an important role in the activation of the innate immune response, which may help to explain the presence of inflammation in people with this disorder. The aim of this study was to analyze the association of *TLR1, TLR2*, and *TLR6* gene polymorphisms in the etiology of schizophrenia.

Methods: We included 582 patients with schizophrenia and 525 healthy controls. Genetic analysis was performed using allelic discrimination with TaqMan probes.

Results: We observed significant differences between patients and controls in the genotype and allele frequencies of *TLR1*/rs4833093 ($\chi2 = 17.3$, $p = 0.0002$; $\chi2 = 15.9$, $p = 0.0001$, respectively) and *TLR2*/rs5743709 ($\chi2 = 29.5$, $p = 0.00001$; $\chi2 = 7.785$, $p = 0.0053$, respectively), and in the allele frequencies of *TLR6*/rs3775073 ($\chi2 = 31.1$, $p = 0.00001$). Finally, we found an interaction between the *TLR1*/rs4833093 and *TLR2*/rs5743709 genes, which increased the risk of developing schizophrenia (OR = 2.29, 95% CI [1.75, 3.01]).

Discussion: Our findings add to the evidence suggesting that the activation of innate immune response might play an important role in the development of schizophrenia.

Keywords: *TLR1*, *TLR2*, *TLR6*, gene–gene interaction, schizophrenia, immune response

Introduction

Schizophrenia is considered a debilitating, complex, and chronic disorder, characterized by the presence of positive, negative, and cognitive symptoms, that affects basic processes such as perception, emotions, and judgment.[1] It is a common disorder, with a lifetime prevalence of 1%; it has been classified as the twelfth most common cause of disability in the world among psychiatric and non-psychiatric illnesses.[2]

The etiology of schizophrenia is unknown; however, there is evidence suggesting that immune alterations could be involved in at least a subset of patients with the disorder.[3] Physiological studies have reported immune alterations in patients with the disorder, such as elevated levels of cytokines and inflammation markers, supporting the immune hypothesis.[4–7] The two-hit hypothesis[8] suggests that a combination of the effects of genetic susceptibility and environmental factors, such as immunological alterations during neurodevelopment, may sensitize the individual to a second stimulus, resulting in the development of schizophrenia-like disease (Figure 1).

Family, twin, and adoption studies have shown genetic and environmental components of schizophrenia, estimating a heritability of 80–85%. Interestingly, genome-wide association studies (GWAS) of the disorder have identified several

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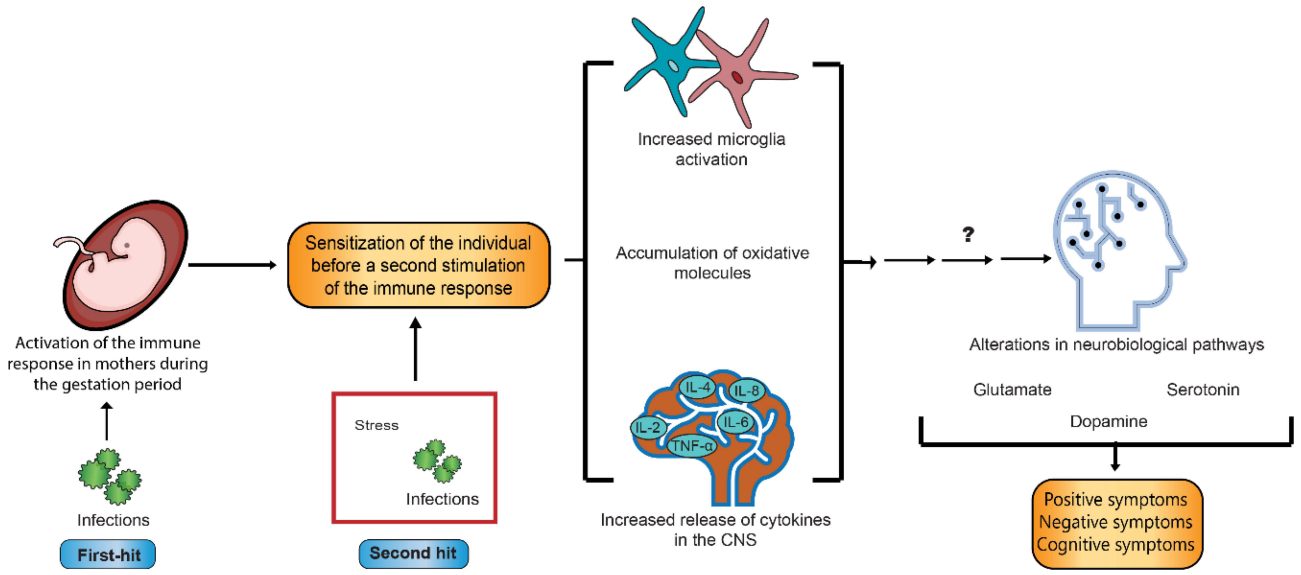

Figure 1 Two-hit hypothesis of inflammation in schizophrenia. During the gestation period of the individual, the mother presents an activation of the immune response during the gestation period, this is known as first-hit. Due to this, the individual is sensitized to a second stimulation of the immune response in later stages of development, which can be caused by stress, bacterial or virus infections, this is known as second hit. This deregulation of the immune system can lead to an increase in microglia activation, an imbalance in the release of cytokines as well as a generation of oxidative stress in the central nervous system (CNS). These conditions can lead to alterations in neurobiological pathways, such as glutamatergic, dopaminergic, and serotonergic pathways producing the characteristic symptoms of schizophrenia.

genes from the immune system.[9–12] An additional GWAS analysis of high-risk genes, including distal genes and those associated with weak GWAS signals, identified overrepresented biologically plausible pathways, showing that the signaling pathway of toll-like receptors (TLR) was overrepresented in schizophrenia.[12]

TLRs are thus possible agents involved in immunological alterations at the level of the central nervous system. Studies carried out in animal models show schizophrenia-like symptoms, including hyperlocomotion, anxiolytic-like behavior, social withdrawal, and cognitive impairment, in *TLR2* knockout mouse brains.[1,13] Specifically, the *TLR1* and *TLR6* genes on chromosome 4p14 and the *TLR2* gene on 4q31.3 have shown lower levels of gene expression in patients with schizophrenia than controls, suggesting a relationship between TLRs and schizophrenia; however, the causes of these alterations remain unknown.[14,15] For this reason, it is important to study TLR1, TLR2, and TLR6 and their association with the etiology of schizophrenia.

Specifically, TLR1, TLR2, and TLR6 belong to the group of receptors located on the cell surface and can form heterodimers, which allow the identification of a wide range of ligands, such as damaged cells, regions of the cell wall of exogenous microorganisms, and the microbiota.[16] In addition, it has been reported that TLRs protect the intestine from potential damage from host bacteria.[17] Also, the intestinal integrity can be affected by changes in the microbiota composition, which can result in bacterial translocation and cytokine synthesis in the systemic circulation; therefore, slowdowns in different neurobiological pathways can increase the risk of developing schizophrenia.[18]

The aim of this study was thus to analyze the association between the *TLR1, TLR2*, and *TLR6* gene polymorphisms and schizophrenia.

Materials and Methods

Sample Selection

This study included 582 Mexican patients, recruited from a schizophrenia clinic from the Instituto Nacional de Psiquiatria Ramon de la Fuente Muñiz, who met the DSM-5 criteria for schizophrenia according to an evaluation with the Spanish version of the Mini International Neuropsychiatric Interview (MINI-5). The study also included a control group of 525 healthy volunteers, also evaluated with the MINI-5 scale, without any diagnosis of mental illness or family history of mental disorders. All participants were Mexican mestizos with a family background of at least three generations born in Mexico, 18 years of age or older, with no chronic immunological diseases. The investigation was

carried out in accordance with the last version of the Declaration of Helsinki. All patients provided written informed consent at time of recruitment and were obtained after the nature of the procedures had been fully explained. The study design was reviewed and approved by the Research Ethics Committee of the Institute (CEI/C/017/2016).

SNP Selection

Three polymorphisms, one each from *TLR1, TLR2*, and *TLR6*, were chosen in accordance with data published in PubMed, the UCSC Genome Browser (human, GRCh/hg38), and the Genetic Association Database.[19] We selected polymorphisms with functionality based on nucleotide changes, location within the gene (regulatory region), or previous association with RNA expression levels, inflammatory processes, or schizophrenia and other psychiatric disorders (Table 1).

Genotyping

Genomic DNA was extracted from peripheral blood samples using the Flexigene DNA kit (Qiagen, Minneapolis, MN). Genotyping was analyzed with custom TaqMan assays for rs4833095 (C_44103606_10), rs5743596 (C_30687147_10), rs4833093 (C_44102593_10), rs3804099 (C_22274563_10), rs7656411 (C_29420880_10), rs5743709 (C_25607738_10), rs5743810 (C_1180648_20), rs3775073 (C_25809256_30), and rs5743827 (C_1180651_20); it was performed with the allele-specific TaqMan assay method, using the ABI Prism®7500 Sequence Detection System according to the manufacturer's protocols (Applied Biosystems Inc., Foster City, CA). The final volume of the reaction was 7 µL, consisting of 100 ng of genomic DNA, 1X TaqMan Universal Master Mix, and 0.71x SNP Genotyping Assay Mix (Applied Biosystems Inc.). After denaturing at 95°C for 1 min, 40 cycles of PCR were performed, denaturing at 95°C for 15s and annealing at 60°C for 1 min.

Statistical Analysis

The power analysis was with QUANTO v.1.2.4 software,[20] with a power of 0.9 to detect a twofold increased risk, assuming an additive genetic model, a risk allele frequency of 0.22, an α level of 0.05, and a control–case ratio of 1:1. Genotype and allele frequency analyses were performed with a chi-square test using Epidat version 3.1.[21] Bonferroni's correction for multiple testing was applied considering the analysis of nine SNPs in the study (0.05/9), adjusting to $p < 0.0055$. A linkage disequilibrium (LD) matrix based on the D' parameter in the patients and controls was estimated using Haploview version 4.2.[22] THESIAS software was used to analyze the haplotype effect. The results were expressed as a haplotypic odds ratio in reference to the most frequent haplotype.[23]

Gene–gene (GXG) interaction was analyzed using Multifactor Dimensionality Reduction (MDR) software version 3.0.2 and MDR Permutation Testing software version 1.0 beta.[24,25] This program is used to detect high-order nonlinear or non-additive interactions in case–control studies with small sample size to improve estimation of the effect of multiple genetic loci in the development of a disease.[23–25] The interaction models are evaluated using testing balanced accuracy (TBA), which measures how often individuals are correctly classified with respect to their case/control status, and cross-validation

Table 1 TLRs Gene Polymorphisms Analyzed in the Study

Gene	SNP	Chr. Position	Gene Position	Major/Minor Alleles
TLR1	rs4833095	4:38798089	Synonymous	C/T
	rs5743596	4:38800907	5′ UTR	A/G
	rs4833093	4:38788119	Intron region	G/T
TLR2	rs3804099	4:153703504	Synonymous	C/T
	rs7656411	4:153706503	3′ UTR	G/T
	rs5743709	4:153705250	Synonymous	A/G
TLR6	rs5743810	4:38828729	Nonsynonymous	A/G
	rs3775073	4:38828211	Synonymous	T/C
	rs5743827	4:38825904	3′ UTR	C/T

Abbreviation: UTR, untranslated region.

consistency (CVC), which evaluates the consistency with which individuals are classified. The best final model is defined for values of TBA of 0.55 to 0.69, a CVC of 10, which is considered strong evidence of a multifactor interaction, and a significant p-value derived from 1000 permutations.[24,26] The MDR software avoids problems in the use of parametric statistics to analyze high-order interactions, minimizes false-positive results due to multiple testing, and does not assume a genetic model.[26]

Results

Demographic and Clinical Characteristics

We include a total sample of 1106 subjects, 581 cases and 525 controls. In the cases sample, 221 (38%) were females and 360 males (62%); and in the control group, 322 (61.3%) were females and 203 (38.7%) males. The mean age and SD in cases was 40.61±2.4 and in the control group was 32.13±11.6. Most of the patients were single (90%), unemployed (72%), and males (63%). Average age and years of education were 40.6±12.4 and 10.25±0.13 years, respectively.

Association Analysis

We observed Hardy Weinberg equilibrium (HWE) for the SNPs *TLR1/* rs4833095, *TLR1*/rs5743596, *TLR1*/rs4833093, *TLR2*/rs3804099, *TLR2*/rs5743709, and *TLR6*/rs5743810 in controls ($p>0.05$). On the other hand, we did not observe HWE for the SNPs *TLR2*/rs7656411, *TLR6*/rs3775073, and *TLR6*/rs5743827 in controls ($p<0.05$). Regarding the patients, the *TLR2*/rs3804099 and *TLR2*/rs7656411 polymorphisms were found in HWE ($p\geq0.05$); we did not find HWE for the variants *TLR1*/rs4833095, *TLR1*/rs5743596, *TLR1*/4833093, TLR2/rs5743709, *TLR6*/rs3775073, *TLR6*/rs5743810, and *TLR6*/rs5743827 ($p<0.05$).

Genotype and allele distributions of *TLR1, TLR2,* and *TLR6* gene polymorphisms are shown in Table 2. There were no differences between the *TLR1*/rs4833095, *TLR1*/rs5743596, and *TLR2*/rs3804099 gene polymorphisms between

Table 2 Genotype and Allele Frequencies of SNPs Analyzed in Patients and Controls

Polymorphism	Genotype			χ 2	*p*	Allele		χ 2	*p*
TLR1									
rs4833095	CC	CT	TT			C	T		
Patients	185 (0.32)	263 (0.45)	133 (0.23)	0.25	0.87	633 (0.54)	529 (0.46)	0.12	0.71
Controls	160 (0.30)	244 (0.46)	121 (0.23)			564 (0.54)	486 (0.46)		
rs5743596	GG	GA	AA			G	A		
Patients	477 (0.82)	94 (0.16)	10 (0.02)	1.2	0.53	1048 (0.90)	114 (0.10)	0.37	0.53
Controls	435 (0.83)	85 (0.16)	5 (0.01)			955 (0.91)	95 (0.09)		
rs4833093	GG	GT	TT			G	T		
Patients	450 (0.77)	108 (0.19)	23 (0.04)	17.3	0.0002	1008 (0.87)	154 (0.13)	15.9	0.0001
Controls	348 (0.66)	149 (0.28)	28 (0.05)			845 (0.80)	205 (0.20)		
TLR2									
rs3804099	TT	TC	CC			T	C		
Patients	286 (0.49)	238 (0.41)	57 (0.10)	1.1	0.56	810 (0.70)	352 (0.30)	0.6	0.44
Controls	253 (0.48)	210 (0.40)	62 (0.12)			716 (0.68)	334 (0.32)		
rs5743709	GG	GA	AA			G	A		
Patients	305 (0.52)	256 (0.44)	20 (0.03)	29.5	0.0001	866 (0.75)	296 (0.25)	7.8	0.005
Controls	342 (0.65)	151 (0.29)	32 (0.06)			835 (0.80)	215 (0.20)		
rs7656411	TT	TG	GG			T	G		
Patients	359 (0.62)	199 (0.34)	23 (0.04)	22.8	0.00001	917 (0.79)	245 (0.21)	12.5	0.0004
Controls	296 (0.57)	169 (0.32)	60 (0.11)			761 (0.72)	289 (0.28)		
TLR6									
rs3775073	TT	TC	CC		0.01	T	C		
Patients	248 (0.43)	232 (0.40)	101 (0.17)	8.0		728 (0.63)	434 (0.37)	31.1	0.00001
Controls	238 (0.45)	227 (0.43)	60 (0.11)			703 (0.67)	347 (0.33)		

(*Continued*)

Table 2 (Continued).

Polymorphism	Genotype			χ^2	p	Allele		χ^2	p
rs5743810	GG	GA	AA		0.86	G	A		
Patients	455 (0.75)	112 (0.19)	14 (0.02)	0.3		1022 (0.88)	140 (0.12)	0.27	0.60
Controls	218 (0.80)	95 (0.18)	12 (0.02)			931 (0.89)	119 (0.11)		
rs5743827	CC	CT	TT		0.01	C	T		
Patients	501 (0.86)	72 (0.12)	8 (0.01)	8.8		1074 (0.92)	88 (0.08)	9.6	0.001
Controls	418 (0.80)	94 (0.18)	13 (0.02)			930 (0.89)	120 (0.11)		

Notes: Bonferroni´s correction adjusted to p-value ≤0.0055.

patients with schizophrenia and controls (Table 2). However, we observed significant differences between patients and controls in genotype and allele frequencies of *TLR1*/rs4833093 ($\chi2 = 17.3$, p = 0.0002; $\chi2 = 15.9$, p = 0.0001; OR = 1.5, 95% CI [1.26, 1.99], respectively), of *TLR2*/rs5743709 ($\chi2 = 29.5$, p = 0.00001; $\chi2 = 7.785$, p = 0.0053; OR = 0.75, 95% CI [0.61, 0.91], respectively), and in the allele frequencies of *TLR6*/rs3775073 ($\chi2 = 31.1$, p = 0.00001; OR = 0.82, 95% CI [0.69, 0.98]) (Table 2).

Haplotype Analysis

Since the variants *TLR2*/rs7656411, *TLR6*/rs3775073, and *TLR6*/rs5743827 were not found in HWE, they were excluded from further analysis. Analysis of the *TLR1* gene indicates that the polymorphic variants are not found in linkage disequilibrium (D' = 0.762, r2 = 0.095; D' = 0.674, $r^2 = 0.04$; D' = 0.745, $r^2 = 0.299$), which indicates that these variants are subject to recombination processes (Figure 2A). Similarly, polymorphic variants of the *TLR2* gene were not found in LD (D' = 0.48, r2 = 0.163), so it was not possible to carry out the haplotype frequency analysis (Figure 2B).

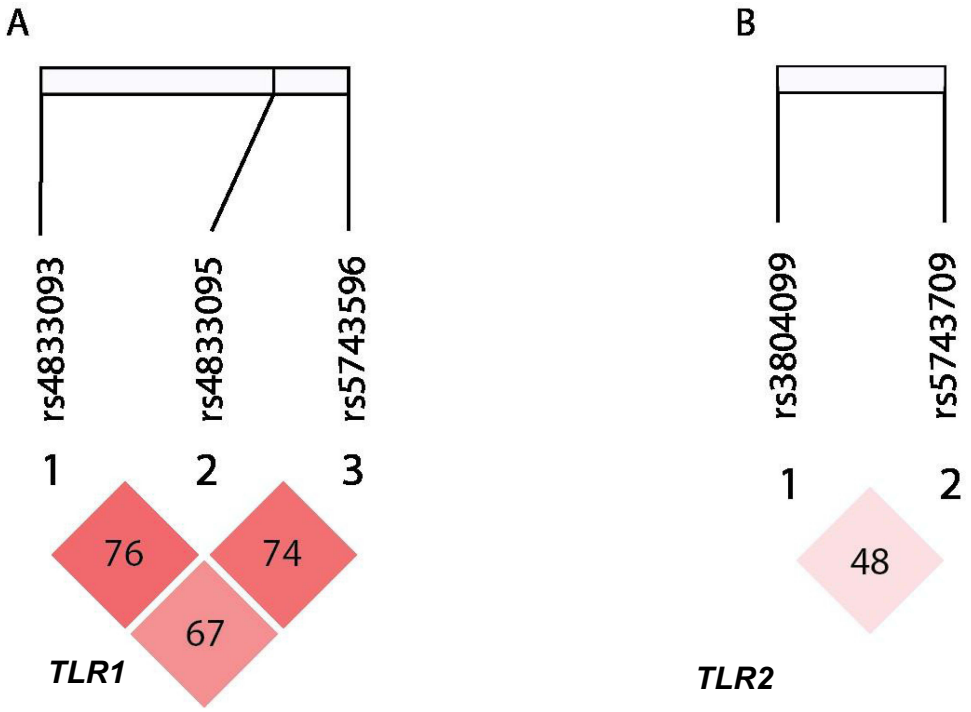

Figure 2 *TLR1* and *TLR2* linkage disequilibrium (LD) structure. The number of the red squares refer to pairwise *D´*. Haplotype blocks were defined using setting of average pairwise D´ within-block of ≥ 0.80, while the color intensity correlates to the pairwise r^2 values as determined using Haploview. (**A**) Linkage disequilibrium structure of *TLR1*. (**B**) Linlage disequilibrium structure of *TLR6*.

Gene–Gene Interaction

Gene–gene interaction analysis showed the best interaction model for *TLR1* and *TLR2* genes with TBA of 0.5824, a maximum CVC of 10/10, $p < 0.0001$, and a 1000-fold permutation test. This result showed a significant interaction between two polymorphic variants of the *TLR1* and *TLR2* genes, reflecting an increased risk in the Mexican population of developing schizophrenia (OR = 2.3, 95% CI [1.75, 3.01] (Table 2)).

Discussion

This study investigated the effect of three candidate genes involved in initiating the innate immune response in a sample of Mexican patients with schizophrenia. The demographic characteristics showed that those with schizophrenia were more likely to be male, have a lower educational level, be single, and be unemployed, which is consistent with data reported in the literature.[27–29]

To our knowledge, this is the first genetic study of *TLR1, TLR2*, and *TLR6* and schizophrenia in Mexican population. Several genome-wide association studies in patients with schizophrenia have shown associations with chromosomal regions containing genes involved in the immune system and inflammation,[11,12,30–32] however only one has shown an association between genes encoding proteins in the signaling cascade initiated by the TLRs.[12]

We find an association of *TLR1*/rs4833093, *TLR2*/rs5743709, and *TLR6*/rs3775073 with schizophrenia. Carriers of the G allele of the *TLR1*/rs4833093 gene polymorphism have a risk of developing schizophrenia that is 1.5 times that of a control group. The G allele of *TLR2*/rs5743709 and T allele of *TLR6*/rs3775073 confer protective effects from schizophrenia.

The *TLR1*/rs4833093 gene polymorphism is located near a regulatory region, which could be altering the expression of the gene,[19] supporting findings of a decreased expression of *TLR1, TLR2*, and *TLR6* genes in patients with schizophrenia, as compared to healthy controls.[15] Studies have found that mRNA stability is affected by the number of optimal and non-optimal codons;[33,34] *TLR2* and *TLR6* variants could thus be related to RNA stability conferring a protective effect from schizophrenia.

In addition, we found no association between the *TLR1*/rs4833095, *TLR1*/rs5743596, or *TLR2*/rs3804099 polymorphisms and schizophrenia, consistent with a study showing no association between *TLR1*/4833095 and schizophrenia in the North Indian population. That study observed an association between *TLR2*/rs3804099 and an increased risk of developing schizophrenia.[35] Oliveira et al reported associations between *TLR2*/rs4696480, *TLR2*/rs3804099, *TLR4*/rs1927914, and *TLR4*/rs11536891 and bipolar disorder, suggesting a pleiotropic effect of TLR genes in psychotic spectrum disorders.[36]

The *TLR1, TLR2*, and *TLR6* gene polymorphisms analyzed in the present study were not found in LD, suggesting that these variants are subject to a recombination process. We also found an epistatic effect between *TLR1*/rs4833093 and *TLR2*/rs5743709, conferring an increased risk for developing schizophrenia. Interestingly, members of the same TLR subfamily tend to form heterodimers to detect their ligands; in particular, *TLR2* forms dimers with *TLR1* to recognize various pathogen-associated molecular patterns (PAMP).[37] The immune activation of TLR in early developmental stages increases the risk of developing such neuropsychiatric disorders.[38] A hypothesis has been formulated about how inflammation may be influencing neurobiological alterations in schizophrenia. This theory is based on immune activation by translocation of bacteria and the synthesis of cytokines in the intestine binding to vagus nerve receptors and reaching the hypothalamus through retrograde neuronal transport in the CNS, which activates microglia, leading to the synthesis of cytokines within the brain. These messengers activate indoleamine 2.3-dioxygenase (IDO1), which is the enzyme in charge of metabolizing tryptophan through the kynurenine pathway, leading to an increase in kynurenic acid, which is associated with alterations in glutamate neurotransmission, which has been associated with the symptomatology of schizophrenia.[4] Therefore, the effect of the interaction of the *TLR1* and *TLR2* variants may result in alterations in neural TLR activation, altering brain function and increasing the risk. The role of TLR activation in individuals with psychotic disorders, however, remains unknown. It has been suggested that damage-associated molecular patterns (DAMP) are involved in the pathophysiology of schizophrenia, but the precise mechanism remains unclear. We hypothesize that the presence of polymorphisms in *TLR1* and *TLR2* genes can modify the affinity between DAMPs and adapter proteins

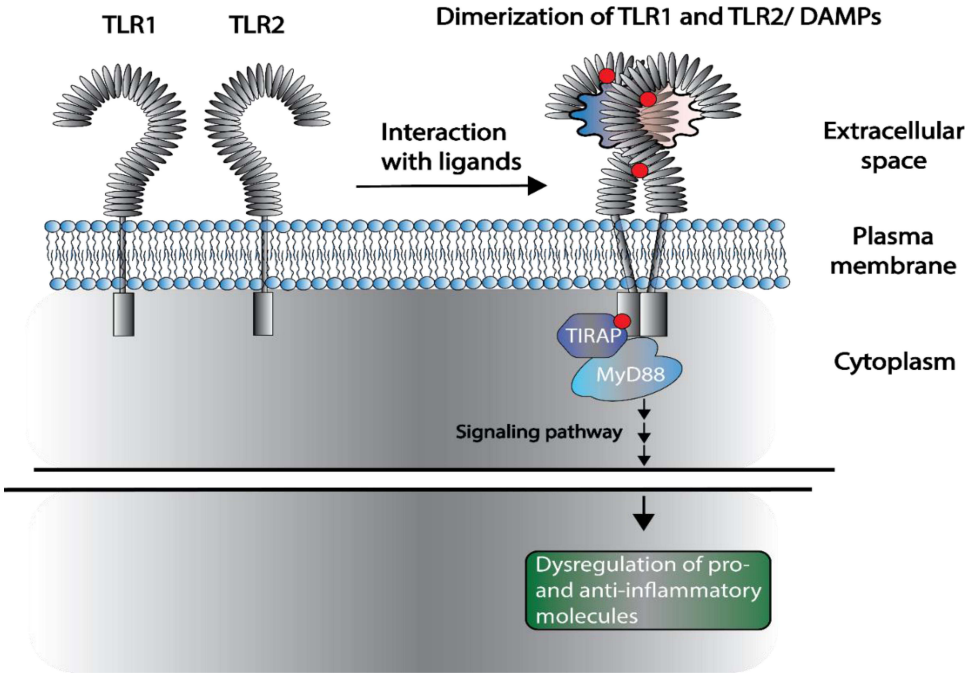

Figure 3 Hypothetical scheme on the effect of SNPs on TLRs. Schematic representation of the formation of TLR1 and TLR2 heterodimers upon interaction with DAMPs. Red dots indicate ligand/receptor binding sites, interaction sites between TLR1 and TLR2, and binding regions with adapter proteins such as the Toll-interleukin-1 Receptor (TIR) domain-containing adaptor protein (TIRAP) and Myeloid differentiation primary response 88 (MYD88). These interactions can be affected by the action of SNPs. We hypothesize that these modifications can trigger deregulations in the levels of pro- and anti-inflammatory molecules, which are altered in patients with schizophrenia.

responsible for activating the signaling cascade of the innate immune response (Figure 3). Preclinical evidence suggests that perinatal infections can trigger an immune and inflammatory response, followed by oxido-nitrosative stress that can lead to behavioral abnormalities in children.[38,39] It would also be interesting to study proteins that participate in the signaling cascade of TLR1, TLR2, and TLR6 receptors, such as myeloid differentiation primary response 88 (MyD88), which has been associated with the etiology of schizophrenia in the Spanish population.[13] There were certain limitations to our study. First, we evaluated only three polymorphisms in each *TLR1, TLR2,* and *TLR6* gene; therefore, the analysis of additional SNPs located in exons or regulatory regions could be important to understand the role of TLR in schizophrenia. Second, we did not match the sex between patients and control group, which could yield important information. Third, our small sample size could not be effective in detecting small genetic effects of *TLR* genes in the etiology of schizophrenia. Fourth, we did not observe HWE in some SNPs analyzed reducing the genetic information in the study. We did not find HWE in the patients group. Interestingly, it has been suggested that a HW disequilibrium might be explained by genetic association showing a link between the locus studied and the disease under study.[40]

It is important to note that because schizophrenia is a multifactorial disorder, with a high comorbidity with other psychiatric disorders, it is difficult to elucidate its etiology.[41] Genetic factors have a major impact on its development; however, gene–environment interaction studies must be carried out to fully characterize its pathophysiology.

Conclusion

Our findings showed an association between *TLR1, TLR2*, and *TLR6* genes and schizophrenia. This suggests that the activation of innate immune response might play an important role in the development of this disorder; however, replication of this study in a larger sample size may help to clarify the role of innate immune genes in the etiology of schizophrenia.

Abbreviations

TLR, Toll-like receptor; GWAS, genome-wide association studies; MINI-5, Mini International Neuropsychiatric Interview; LD, linkage disequilibrium; GXG, Gene–gene interaction; MDR, Multifactor Dimensionality Reduction;

TBA, testing balanced accuracy; CVC, cross-validation consistency; OR, odd radio; PAMP, pathogen-associated molecular patterns; DAMP, damage-associated molecular patterns; MyD88, myeloid differentiation primary response 88; CNS, central nervous system; TIR, Toll-interleukin-1; TIRAP, TIR domain-containing adaptor protein.

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Disclosure

The authors report no conflicts of interest in this work.

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