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Original Article

TLR8 is highly conserved among the Saudi population and its mutations have no effect on the severity of COVID-19 symptoms

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Abstract: Coronavirus 2019 (COVID-19) is an infection caused by the newly discovered severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The innate system is the first line of defense against pathogens and diverse infectious agents. It has been suggested to play a key role in the development of the cytokine storm and promoting other severe forms of chronic inflammation. Toll-like receptors (TLRs) are crucial for the innate immune response to pathogens. TLR8 is expressed on myeloid cells and phagocytes, where it acts as an endosomal sensor of RNA degradation. The present study aimed to investigate whether the severity of COVID-19 symptoms could be associated with certain genetic variations of TLR8. We collected blood samples from 45 participants who had moderate to severe respiratory symptoms and a positive COVID-19 PCR test result within 3-5 days of sample collection. Genomic DNA was extracted from the blood samples, then exon 2 of the TLR8 gene was amplified with polymerase chain reaction (PCR), and PCR products were utilized for sequencing. DNA sequencing showed an average of 99.63% sequence homology in TLR8 across all samples. Base-pair homology analysis revealed variations in TLR8 at two positions: X:12937804 (rs5744080) and X:12937513 (rs2159377). The results revealed that these two mutations had no detrimental effect on symptoms in the target population. Our results show that specific SNPs did not affect the final receptor function of TLR8. This finding also indicates that the innate immune response, once activated, does not depend on the innate immune receptor's level of affinity for identifying their respective glycoprotein structures on the SARS-CoV-2 virus.

Keywords: COVID-19, immunology, TLR8, toll-like receptors, DNA, mutation, symptoms, SARS-CoV-2

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel type of coronavirus that causes coronavirus 19 (COVID-19) disease, which is a positive-sense single-stranded (ss)RNA virus that belongs to the coronavirus family [1]. This virus caused an outbreak in Wuhan, China, in late 2019, and COVID-19 was subsequently recognized as a pandemic by the World Health Organization in March 2020 [2].

The innate immune system is the first line of defense against pathogens and diverse infectious agents and plays an essential role in the development of cytokine storms and promoting other severe forms of chronic inflammation and cancer [3]. Thus, it is of great importance to further study this arm of the immune system to improve the current understanding of disease pathogenesis [4, 5].

The innate immune response comprises Toll-like receptors (TLRs), which are important receptors involved in response to diverse types of pathogens. The host's innate immune system encounters microorganisms and responds to their stimuli primarily via TLR recognition [6]. The activation of TLRs enhances intracellular signaling pathways that result in the release of inflammatory cytokines, type I interferons, and chemokines [7, 8], which ultimately leads to the stimulation of the immune responses required to abolish the pathogen [9, 10]. It has been reported that TLR3, -7, -8, and -9 are expressed on intracellular vesicular membranes, where they are involved in the recognition of nucleic acids [11]. Moreover, TLR8 is highly expressed

in monocytes, macrophages, and myeloid dendritic cells (DCs) [12]. Pathogen-associated molecular patterns (PAMPs) are known to drive the majority of immune responses, as inflammatory reactions are initiated after the innate arm of the immune system senses the presence of PAMPs [13, 14]. TLR8 is an endosomal sensor of RNA degradation in human phagocytes, which is activated upon recognition of viral and bacterial pathogens [15]. However, although the role of TLR8 is known to be comprehensive, the exact effect of TLR8 throughout the course of infection remains poorly defined [16]. TLR7 and TLR8 detect ssRNA, while TLR9 responds to double-stranded DNA viruses by identifying non-methylated viral CpG-comprising DNA [17, 18]. Although the majority of TLRs sense pathogen constituents on the cell surface, intracellular TLRs, such as TLR8, sense DNA or RNA molecules in the cytoplasm [19, 20]. Myeloid cells, along with certain human plasmacytoid DCs, express TLR8 [21]. Previous bioinformatics (16) studies on SARS-CoV infection have established that certain parts of the viral genome are associated with the immunostimulant action of tumor necrosis factor-α and interleukin (IL)-6, as well as IL-12 stimulation, via TLR7 and TLR8, where the response of these inflammatory factors was nearly two-fold greater than other ssRNA viruses [19].

T cells play an essential role in the anti-SARS CoV-2 response by promoting the secretion of $CD4^+$ T cell-derived interferon gamma (IFN)-γ and $CD8^+$ T cell-mediated cytotoxicity, in addition to stimulating B cell antibody production [22]. SARS CoV-2 has been found to evade these mechanisms via the stimulation of T cell apoptosis [21]. Nonetheless, T cells may not only become depleted because SARS CoV-2 stimulates their apoptosis but also because they may recognize the proinflammatory cytokines expressed by the innate immune inflammatory cells of the lung and localize to the lungs, eliciting hyperinflammation and triggering a cytokine storm [22].

Patients and methods

Ethics statement

The present study was approved by the Internal Review Board of the General Directorate of Health Affairs in Madinah, Saudi Arabia (approval no. IRB496). Written informed consent was obtained from each patient prior to participation in the study.

Study participants

The study participants comprised 45 Saudi inpatients, 27 males and 18 females, aged between 38 and 67 years, and were admitted to Ohoud hospital in Madinah between May and August of 2020. All participants had moderate to severe respiratory symptoms and had received a positive COVID-19 polymerase chain reaction (PCR) test result within 3-5 days of sample collection. Patients with no PCR confirmation or who presented with acute symptoms were excluded from this study. In total, 3 ml venous whole blood was collected and processed for genomic DNA (gDNA) extraction.

DNA extraction

gDNA was extracted from venous whole blood using a gSYNC DNA Extraction Kit (Geneaid Biotech Inc., Taipei), according to the manufacturer's protocol. Briefly, approximately 200 µL GST buffer and 20 µL proteinase K were added to 200 µL blood and incubated for 15 minutes at 60°C. The mixture was subsequently centrifuged at 16,000×*g* for 2 minutes, and the supernatant was transferred to a new 1.5 ml microcentrifuge tube. Then, 200 µL GSB was added to each tube and mixed gently for 10 seconds, prior to the addition of 200 µL absolute ethanol to the mixture. The entire content was subsequently transferred to GS columns and centrifuged at 16,000×g for 2 minutes. The gDNA was washed twice with 400 µL and 600 µL W1 buffer, and 50 µL pre-heated elution buffer was then added to the GS columns and left to stand for 3 minutes at room temperature to allow for complete absorption of the elution buffer. DNA was finally eluted from the GS columns by centrifuging the columns at 16,000×g for 30 seconds. The DNA concentration and purity of the extracted samples were determined by analyzing 1 µL elute using a NanoDrop 1000 UV-VIS Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

DNA sequencing

Polymerase chain reaction (PCR) was performed with 20 µL reaction volumes using 3 ng

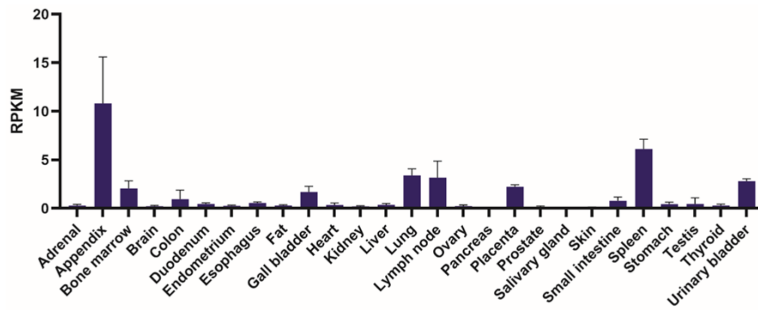

Figure 1. RNA sequencing data from 27 different tissues from 95 individuals. RNA-seq data indicate that TLR8 is highly expressed in secondary lymphoid organs.

DNA and 10 pmol of each oligonucleotide (forward, 5'-GCCTGTAACTCTATACCCATGTC-3' and reverse, 5'-TAATACCCAAGTTGATAGTCGATAAGTTTG-3') on a Veriti 96-Well Thermal cycler (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The following thermocycling conditions were used for the PCR: 92°C for 2 minutes, followed by 40 cycles of 92°C for 15 seconds and 60°C for 1 minute. Subsequently, 1 μL labeled PCR products were loaded and mixed with formamide (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and a ROX-labeled GS500-LIZ-3730 standard (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). DNA fragments were denatured and size-fractionated using capillary electrophoresis on an ABI 3730 automatic DNA sequencer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). DNA sequencing was performed using genotyping via capillary electrophoresis on an ABI PRISM 3730 analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Statistical analysis

RNA-sequencing (RNA-seq) data for ensg00000101916 (TLR8) were obtained from "The Expression Atlas" (The European Bioinformatics Institute, Wellcome Genome Campus, Hinxton, Cambridgeshire). RNA-seq datasets from various organs and tissues were graphed as reads per kilobase million. gDNA sequencing files were visualized and assessed for quality using Geneious Prime software (version 2021) and then aligned against the TLR8 sequence from the Genome Reference Consortium Human Build 38 patch release 13 (GRCh38.p13) [23] using the pairwise multiple align function. Single-nucleotide polymorphism (SNP) data, including reference nomenclature, haplotyping and allele frequency, were obtained from Ensembl genome browser (release 103, EMBL-EBI) [24]. Individual SNP data were plotted and graphed using GraphPad Prism software (version 7.2; GraphPad Software, Inc., La Jolla, CA, USA) as grouped columns to express the percentage of each genotype in the different populations.

Results

TLR8 expression is upregulated in secondary lymphoid organs

RNA-seq data from tissue samples representing 27 different tissues from 95 individuals were used. As shown in **Figure 1**, TLR8 expression was found to be upregulated in the appendix, spleen, lymph nodes and other organs that are exposed to external environmental factors.

TLR8 is highly conserved with minimal variations in the targeted population

The second exon of TLR8 was targeted with specific sequencing primers. DNA sequencing identified an average of 99.63% sequence homology in TLR8 between the 45 samples. Reviewing the sequence map of ensg00000101916 between X:12,937,163 and X: 12,941,288 revealed 573 possible DNA variations. Our base-pair homology analysis using the Geneious prime software (Geneious Alignment; 65% similarity; gap open penalty: 12; gap extension penalty: 3) found variations at only two positions: X:12937804 (rs5744080) and X:12937513 (rs2159377) (**Figure 2**). The reference genome reported a homozygous C for rs2159377 and a homozygous T for rs5744080. For the rs2159377 mutation, the results showed that the allele frequencies were 13, 6 and 26 for CC, CT and TT, respectively. We then compared the allele frequency of our target population with other population using data from the "Population Genetics" function of the Ensembl database (release 103, EMBL-EBI). This distribution was similar to that found in East Asian, South Asian and North American

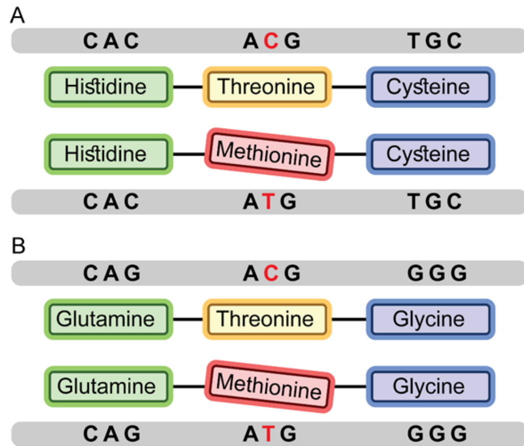

Figure 2. Visual representation of the DNA variants showing the reference DNA and amino acid sequence (top) and the reported variant and amino acid change (bottom). A. DNA variant rs2159377; B. DNA variant rs5744080.

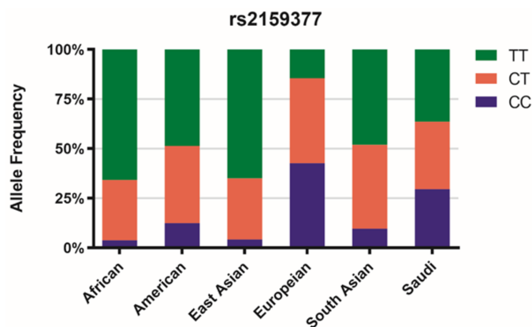

Figure 3. Allele frequency of the DNA variant rs2159377 at position X:12937513 of the *TLR8* gene. This distribution shows that Saudis have a similar distribution to East Asians, South Asians, and Americans.

populations (**Figure 3**). The rs5744080 mutation had allele frequencies of 13, 15 and 17 for CC, CT and TT, respectively. This distribution was similar to that of East Asian and North American populations (**Figure 4**).

Targeted TLR8 variations are not associated with COVID-19 severity

The present study target population was Saudi patients with moderate to severe respiratory symptoms that had received a positive PCR result for COVID-19 infection within 3 days of sample collection. The allele frequency for both targeted SNPs (rs5744080 and rs2159377) was relatively broad and nondetrimental.

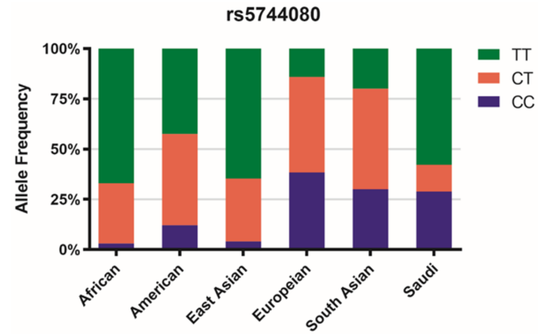

Figure 4. Allele frequency of the DNA variant rs5744080at position X:12937804 of the *TLR8* gene. This distribution shows that Saudis have a similar distribution to East Asians and Americans.

Discussion

It has been previously shown that TLR8 has an important role in sensing ssRNA viruses, such as the SAS-CoV-2, by recruiting MyD88 and activating transcription factor NF-κB and initiating the antiviral immune response [23]. The present RNA-seq data revealed that TLR8 expression was upregulated in several secondary lymphoid organs and tissues. The upregulated expression of TLR8 in these tissues suggests the importance of TLR8 in the process of innate immunity. Antigen-presenting cells use lymphatic vessels to migrate to primary and secondary lymphoid organs, where they can interact with naïve T cells to help identify pathogens [24]. This indicates that the innate immune system depends on TLRs, such as TLR8, to identify internal and external pathogens through their conserved structures. Since PAMPs are conserved, the structures that identify them (such as TLRs) are also considered to be conserved. Mutations in genes responsible for producing immune receptors pose a significant biological challenge, as alterations in the amino acid sequence may affect the overall protein function, thereby hindering the immunological ability of the protein.

Most SNPs in the human genome have no detrimental effect on the overall protein function [25]. However, occasionally, single mutations in genes may have a significant role in predicting protein function or interactions with other molecules [26]. The present study identified two distinct SNPs in exon 2 of the TLR8 mRNA, namely rs5744080 and rs2159377; however, neither was found to have an effect on the severity of symptoms in the COVID-19 patients.

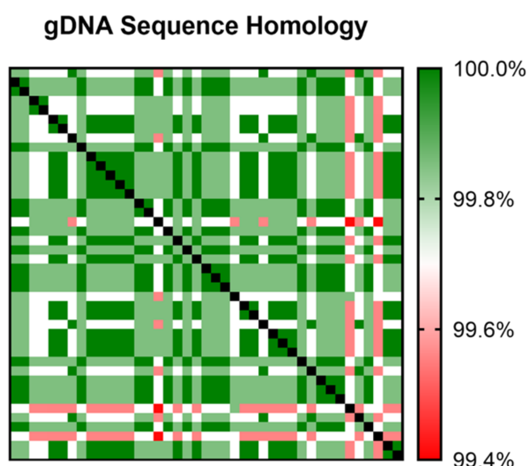

Figure 5. DNA sequencing homology of exon 2 of the *TLR8* gene from 45 samples.

After reviewing the DNA sequence of TLR8 (ensg00000101916) between X:12,937,163 and X:12,941,288, we identified 573 possible DNA variations. Our base-pair homology analysis subsequently found variations in only two positions: X:12937804 (rs5744080) and X: 12937513 (rs2159377) in the present study target population. DNA sequencing of exon 2 of the TLR8 mRNA revealed more than 99.4% sequence homology in our target population (**Figure 5**). This suggests that TLR8 is highly conserved in our target population, with minimal changes from the reference human assembly, GRCh37.

The rs2159377 mutation had a broad allelic distribution between heterozygous CT, homozygous CC or homozygous TT. Conversely, the rs5744080 mutation showed a preference for homozygous T, followed by homozygous C. Notably, both DNA variations resulted from a substitution of methionine for threonine.

Significant research has been conducted to determine the association between the identification mechanism of the innate immune system and the subsequent immune response [27]. Numerous previous studies have suggested that viral loads in symptomatic patients are directly associated with the severity of such symptoms [28]. However, to the best of our knowledge, no previous studies have investigated the interaction between the actual mechanism used by the innate immune system and its associated receptors, and whether changes in the molecular structures of these receptors may consequently affect the severity of symptoms of diseases.

The findings of the present study determined that specific SNPs did not affect the final receptor function of TLR8. Although the two reported variations were synonymous and would change the molecular structure of TLR8, the severity of COVID-19 symptoms was not affected. These findings indicates that the innate immune response, once activated, does not depend on the level of affinity of the innate immune receptor for identifying their respective glycoprotein structures on the SARS-CoV-2 virus. Further functional studies are required to determine the effect of the identified DNA variations on their downstream signaling targets. However, our study showed that these two mutations had no detrimental effect on our target population's symptoms.

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Disclosure of conflict of interest

None.

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