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Prediction of deleterious non-synonymous SNPs of human TLR2 gene associated with bacterial meningitis and hearing impairment by computational approach

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Abstract---Background: Bacterial meningitis is the most serious infection of the central nervous system and often occurs in children under the age of 5 years and leads to hearing loss, learning disabilities. Several studies have reported the genetic variation in a gene that is involved in the innate immune system and affects the susceptibility, severity of bacterial infection during meningitis. Methodology: Hence the present study was performed to insights the single nucleotide polymorphisms in pathogen recognition genes especially those that play an essential role in the innate immune system. Toll-like receptor 2 (TLR2) was selected to analyze the association of TLR2 in bacterial meningitis. The coding and non-coding SNPs of the human TLR2 gene was retrieved from the NCBI database and were subjected to various computational prediction tools including SIFT, PolyPhen-2, Imutnat, to predict the high-risk deleterious SNPs and the effect of single amino acid substitutions and structural and functional impact of these mutations was predicted with project HOPE, SNP-GO tools. Results: The result revealed that among the 501 non-synonymous, 9 deleterious SNPs were sorted by SFIT, Polyphen-2 and I mutant serve with SFIT score, DDG score, and PISC score which are equal to 1. To understand the recognition mechanism of bacterial peptides by TLR2, peptides docking was also performed. Conclusion: Based on the current findings, it can be concluded that the SNPs (rs5743708, rs121917864, and rs371652530) were predicted as important candidates. This data may contribute to the understanding of bacterial meningitis and may help to develop effective antibiotics for severe infectious diseases and bacterial meningitis.

Keywords---bacterial meningitis, TLR2, gram-positive bacteria, SNPs, SFIT, Polyphen-2, I-mutant, peptide docking.

Introduction

The bacterial infection meninges is one of the critical and emergency diseases commonly caused by *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* type b in children (Chávez-Bueno and McCracken, 2005). The infection of bacterial meningitis is characterized by inflammation and results in significant mortality worldwide (Mook-Kanamori et al., 2011). It causes 50% of a patient's death if untreated. It can be diagnosed and treated adequately within 24 to 48 hours of symptoms onset can reduce the death of patients up to 8- 15 %. However, 10- 20% of the patients have an impact such as brain damage, hearing loss, and learning disability (Mook-Kanamori et al., 2011). Due to no proper treatment and vaccination, the mortality of bacterial meninges approaches 100% (Kim, 2010). Hence prompt proper and accurate diagnosis and treatment the identification of effective antibiotics is essential with adequate antibiotic therapy.

The universal use of most of the conjugated vaccines are effectively targeting the *S. pneumoniae*, *N. meningitidis* shows the high incidence of bacterial meningitis it shows highest case rates in 2009; it was estimated that 80, 000 and more than 4000 deaths. The etiological agents are highly responsible for bacterial meningitis that vary by different age groups (Thigpen et al., 2011; Oordt-Speets et al., 2018). In China, it was estimated that 6.95 to 22.3% cases were reported in 100, 000 population during 2006-2009 under the age group of 5 years (Li et al., 2014), and the fatality rate was estimated up to 18.42% for the children under the age group of 5 years (Dong et al., 2004). However, there was a sharp rise in the mortality and death in penicillin resistant *S. pneumoniae* during those years (Yao and Zhang, 2011). Therefore the understanding of the mechanism of bacterial interaction remains a challenge.

In host organisms, pathogen recognition receptors are present in various cells including microglia and astrocytes inside the central nervous system (Mogensen, 2009), which recognize pathogen-associated molecular patterns. The high binding affinity activates the nuclear factor kappa B and subsequent genetic transcription and pro-inflammatory cytokines. Among them, Toll-like receptors (TLRs) is one of the major groups of pathogen recognition receptors and a type of transmembrane protein and engages the various proteins including kinase protein IL-1R1-associated protein kinase (IRAK), transforming growth factor-beta activated factor (TAK1) (Sorrentino et al., 2008; Akira et al., 2006). The site of TLR stimulation during the infection results in the production of cytokines and chemokines hence it plays a crucial role in the initiation of inflammatory response against pathogens like *S. pneumoniae* and *N. meningitidis*. In 2002, Echchannaoui and their co-workers first reported the role of TLRs in pneumococcal meningitis; this study reported the more invasive course of pneumococcal infection in TLR2-deficient mice. The other TLRs such as TLR4 and TLR9 also have been reported in the inflammatory response induced by the presence of live bacterial *S. pneumoniae* and *N. meningitidis* (Ribes et al., 2010; Wright et al., 2009; Brouwer et al., 2010). The binding of viral cell wall protein of *S. pneumoniae* such as lipoteichoic acid

and porin B to the TLR2 also activates the production of inflammatory cytokines (Sanders et al., 2011; Massari et al., 2006).

Single nucleotide polymorphisms (SNPs) are a recently widely used study to evaluate the most common genetic variation in populations. So far, 500,000 SNPs have been identified that are responsible for coding various genetic associated diseases. Among them, non-synonymous SNPs (nsSNPs) significantly lead the amino acid changes and reduce the gene product and influence the immunological response to drugs (Reich et al., 2003; Marnellos et al., 2003). Numbers of in vitro studies have also been reported the certain SNPs and their associated mutation have a substantial effect on the susceptibility to and severity of invasive bacterial infections. For example, the functional polymorphism in the TLR4 gene (rs4986790; D896G) has conformational changes which result in the reduction in surface expression and also affecting folding efficacy and stability off MyD88 and their downstream messengers' interaction (Toshchakov et al., 2002; Faber et al., 2009). Hence in the current study, we are aimed to investigate the crucial SNPs of TLR2 and their impact on bacterial meningitis with various silico approaches.

Materials and Methods

The SNP data set of the TLR2 gene was retrieved from the National Center for Biological Information (NCBI) Single Nucleotide Polymorphism (SNP) database. The SNP related ensembles proteins were retrieved from Uniprot databases (Alwi, 2005). The coding and non-synonymous SNPs were selected and targeted for various analyses by computational software.

Prediction of deleterious SNPs by SIFT analysis

SIFT (sorting intolerant from tolerant) is a widely used online tool for the prediction of both deleterious and tolerated SNPs. It effectively detects the influence of amino acid substitution on a protein which leads to function and phenotype alterations. It is a multistep homology-based online tool which generates the alignments of multiple homologous proteins to assign score for every residue and depict the score ranging from 0 to 1 (Warde-Farley et al., 2010; Ng et al., 2003). The rsIDs of SNPs were utilized as input in this tool, the results show the sorting intolerant from tolerant SNPs. Available at http://sift.dna.org/www/SIFT_dbSNP.html.

Polymerphism phenotyping (polyphen-2)

To evaluate the functional changes in the deleterious SNPs Polyphen-2 bioinformatics software was used in this study. It automatically predicts the impact of amino acid substitution and protein function, hence this step is crucial to insights the functional changes in the protein. The amino acid sequence was used and the input query, based on the sequence and effect of amino acid changes on the protein and phylogeny has been used for the prediction; it also considers the multiple sequence alignment of the three-dimensional structure of the protein. It correlates this multiple sequence alignment with different protein structure and calculates the position-specific independent count (PSIC) score

ranging from 0 to 1. The range of PSIC scores categories the SNPs as benign (0-0.2), possible damaging (0.2-0.85), and probably damaging (0.85-1) (22) (Ramensky et al., 2002; Vanajothi et al., 2012; Bhavaniramya et al., 2019). (Available at: <http://genetics.bwh.harvard.edu/pph>).

I-Mutant

To find the stability of the amino acid change in the single-site mutation, I mutant (<http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi>) was used. It significantly estimates the free energy changes by computing Gibbs free energy of wild protein and subtracting it from the mutant type. In this study, the deleterious SNPs of TLR2 were submitted as input to the I-mutant server to predict the stability of the amino acid changes and free energy changes in the terms of reliability index value (RI) (Venselaar et al., 2010; Rajamanikandan et al., 2012).

SNPs and Go

SNP and Go (<http://snps.biofold.org/snpsand-go//snps-and-go.html>) predict disease-related polymorphism and its related mutations in protein structure. The FASTA sequence of the protein was used as input and the results were depicted based on the prediction of the probability of the score ranging from 0 to 0.5. The higher value 0.5 and above reveals the diseased related effect of the mutation.

Project HOPE

HOPE (<http://www.cmbi.ru.nl/hope/home>) is the widely used web server for the evaluation of structural modification and its intended amino acid substitution. It uses Uniprot and DAS prediction servers and provides the structural information of both wild and mutant type residues and also results in the 3D structures of the mutated proteins.

Analysis of amino acid evolutionary conservation

Consurf (<http://consurf.tau.ac.il/>) is widely used to analyze the evolutionarily conserved amino acid residues. It determines the highly conserved amino acid residues using the empirical Bayesian method. The amino acid sequence of TLR2 was utilized as input to the Consurf server to identify both structural and functional residues in the evolutionarily conserved region. The output of the Consurf server depicts the conservation score and color indication based on the highly conserved residues, the score range between 1-4 represent the variable, range between 5-6 represent the intermediate, and range between 7-9 represent the conserved amino acids (Landau et al., 2005).

Identification of TLR2 gene and subnetwork analysis

The protein-protein interaction (PPI) network of the TLR2 gene with other major proteins involved in immunological response during bacterial infection was built by the Search Tool for the Retrieval of Interacting genes (STRING) software.

Retrieval TLR2 Protein and viral peptides

The 3D structure of TLR2 (PDB ID: 1FYW) was retrieved from the Protein Data bank (PDB) with a resolution of 2.80 Å. All the peptides sequence was retrieved from peptide bank (<http://pepbank.mgh.harvard.edu/downloads>). From the literature six bacterial peptides show significant binding interaction with TLR2 during bacterial infection, hence those seven peptides were selected for protein-peptide docking. The structures were manually visualized in Pymol software.

Molecular docking

The interaction of TLR2 with bacterial origin peptides was evaluated by HDOCK software. Molecular docking is one of the widely used and powerful tools to find the binding affinity of the protein-ligand and protein-protein complexes. Docking programs generate the conformations of the protein-ligand complex and then rank the conformation with the scoring function. The chain A of TLR2 was used as a receptor molecule and the bacterial peptide was used as ligand molecules for pepdocking (de Vries et al., 2010). Molecular docking was performed by using the HEPDOCK web server with default parameters to dock peptides and protein molecules. The chain A of TLR2 was given as input in HEPDOCK; the 3D structure of a sequence of the peptides was generated with the MODPEP program. The HEPDOCK works via a hierarchical algorithm for both flexible and blind docking. Based on the generated pose the binding affinity of the peptide with TLR2 was ranked by binding energies of each docking pose. The best docking pose was selected based on the score. To evaluate the binding affinity of bacterial peptides with TLR2, the site-directed mutation was done by using a Swiss PDB viewer and the mutated protein was again docked with six bacterial peptides.

Molecular docking data analysis

A protein-protein check (PP Check) web server is used to examine the interactions for the selected docking complex. The key interactions such as the formation of hydrogen bonds (H-bonds), salt bridges, and the hydrophobic interactions that occur upon docking peptidoglycan peptides lengths of both wild type and mutant type TLR2 were examined.

Results and Discussion

Prediction of deleterious SNPs in the coding region by SIFT and Polyphen-2

Exploring the desired gene using dbSNPs/NCBI TLR2 gene was studied in the NCBI database ([http:// www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)). A total of 6907 SNPs were present in Homo sapiens, among them 107 were found in coding non-synonymous regions (missense) and 270 and 104 were synonymous with 3' prime and 5' prime regions respectively. All the non-synonymous, 3' prime, and 5' prime region SNPs were analyzed by SIFT software. Out of 501 SNPs were given as inputs to SIFT software among them nine SNPs were predicted to be deleterious (Table 1). From the result of the Polyphen-2 server, it was predicted that among the nine SNPs about seven SNPs (rs5743708, rs121917864, rs5743704, rs5743706, rs370330063, rs371652530, and rs37272192) found to be probably

damaging using Polyphen-2 server with a high score of 1.000 and the remaining 2 SNPs (rs139227237 and rs143997997) shows 0.985 and 0.991 and come under the category of probably damaging SNPs. Bhavaniramya et al (2020) reported that TLR2 deleterious SNPs of bovine which are associated with bacterial infection with experimentally proved SNPs and the results were predicted as deleterious and probably damaging with a score of 1.000 for all the experimentally proved SNPs. The study of van Well et al. (2013), reported the single nucleotide polymorphisms in TLR2 via experimental studies and results revealed that the SNP rs5743708 was predicted as deleterious one and the mutation in this SNP play a crucial role in susceptibility to the severity of the bacterial infection. In this study also we have predicted that rs5743708 a deleterious one and is found to be probably damaged by SIFT and Polyphen-2 respectively.

Table 1 Nonsynonymous SNPs predicted with SIFT, Polyphen, and I-Mutant, selected SNPs with PSIC SD range (1–0.99) and Tolerance Index equal (0.009)

S.NO	SNPs	SIFT score	AA change	PICS score - PolyPhen-2	DGC Value (I-Mutant)
1	rs5743708	0.016	R753Q	PROBABLY DAMAGING (1.000)	-0.93 Kcal/mol
2	rs121917864	0.001	R677W	PROBABLY DAMAGING (1.000)	-0.30 Kcal/mol
3	rs139227237	0.005	F217S	PROBABLY DAMAGING (0.985)	-1.61 Kcal/mol
4	rs5743704	0.002	P631H	PROBABLY DAMAGING (1.000)	-1.05 Kcal/mol
5	rs5743706	0.001	Y715N	PROBABLY DAMAGING (1.000)	-1.31 Kcal/mol
6	rs143997997	0.006	P499S	PROBABLY DAMAGING (0.991)	-1.33 Kcal/mol
7	rs370330063	0.002	R748H	PROBABLY DAMAGING (1.000)	-1.18 Kcal/mol
8	rs371652530	0.001	R723C	PROBABLY DAMAGING (1.000)	-0.97 Kcal/mol
9	rs372721923	0.001	Y297C	PROBABLY DAMAGING (1.000)	-1.16 Kcal/mol

Prediction of amino acid change in stability due to mutation using I-Mutant

All the 99 SNPs that were predicted from SIFT and Polyphen-2 software as deleterious and probably damaging were submitted to I-mutant to predict all the possible mutations in the TLR2 gene and the results revealed that the score values (DDG) > -1.0 Kcal/mol (rs139227237, rs5743704, rs5743706, rs143997997, rs370330063, and rs372721923) indicates those SNPs are less stable the given point mutation (Table 1) and the experimentally proved SNP rs5743708 (- 0.93 Kcal/mol) also show more or less close to the value of -1.0 Kcal/mol. Based on the results it was predicted that all the nine predicted SNPs were seen to be most deleterious, and found to be probably damaging. The

outcomes predicted all the SNPs and their associated mutations in the TLR2 gene decreased the stability of the protein.

Determination of probability scores of predicted SNPs using SNPs and Go software

The SNPs predicted from SFIT and Polyphen-2 were selected and submitted to the SNPs and Go software to find the diseases related to deleterious SNPs. The results of SNPs and Go software revealed that all the nine predicted deleterious SNPs are found to be disease-related with RI score ranging from 5 to 8 and are illustrated in table 2.

Table 2 Nonsynonymous SNPs predicted with SNPs & Go programs, the selected SNPs shows the RI score and the range of probability

S. No	SNP	Mutation	Prediction	RI Score	Probability
1	rs5743708	R753Q	Disease	5	0.753
2	rs121917864	R677W	Disease	8	0.877
3	rs5743706	Y715N	Disease	4	0.722
4	rs370330063	R748H	Neutral	3	0.346
5	rs371652530	R723C	Disease	7	0.844

Findings of project HOPE software

Based on the results of SFIT, Polyphen-2, I-mutant, and SNPs-GO the highly deleterious and damaging SNPs such as rs5743708, rs121917864, and rs371652530 were selected and submitted to Project HOPE to finding the substitution of amino acids in the wild type protein, the results revealed that the experimentally proved SNP rs5743708 showed substitution of Arginine (wild type) to Glutamine (mutant) at the position of 753. The mutant residue is smaller than wild-type residue and neutral charge, and more hydrophobic than wild type residue. The result of rs121917864 showed the substitution of Arginine (wild type) to tryptophan (mutant type) at the position of 677. In this substitution, the mutant type residue is bigger than the wild type residues and has the neutral charge while the wild type has a positive charge, compared to wild type residue the mutant types are more hydrophobic and make contact with residues located in the structural domain. The deleterious SNPs rs371652530 showed the substitution of Arginine (wild type) to Cysteine (mutant) at the position of 723. The mutant residue Cysteine is smaller than wild type residue and has the neutral charge, the wild type residues are positively charged. Compared to Arginine the mutant type residue is a more hydrophobic nature and makes contact with structural domain residues. Overall all these three mutant residues were located near the conserved region of TLR2 protein and also all these three amino acid substitutions occurred in the TIR domain of TLR2. The difference in hydrophobicity and charge of the residues may affect the hydrogen bond formation and may disturb the ionic interaction made by wild-type residue Arginine. This amino acid substitution can also disturb the protein function and disturb the interaction of other small molecules and leads to pathogenicity. The amino acid substitutions of three SNPs were illustrated in Figure 1.

Analysis of amino acid evolutionary conservation

The changes in the conserved region and the evolutionary profile of the TLR2 gene were predicted by Consurf software. The result of the Consurf analysis showed that most of the amino acid residues are located in a conserved region with a score ranging from 7-9. The amino acids involved in mutation such as R753Q, R677W, and R723C were predicted as highly conserved residues with a color range of 9. Figure 3 shows the conserved residues of predicted results of ConSurf of TLR2 based on the 9 color variation the amino acid residues were predicated as a variable, average and highly conserved region and functional residues. This evolutionary profile predicts the amino acid substitution and the deleterious effect of desired gene interest (Ashkenazy et al., 010; Ramensky et al., 2002). The conservation scale was represented as e, b, f, and s, in the predicted results all the three amino acids which are involved in possible amino acid substitution were located in a highly conserved region with conservation scale "f". The results indicate the three SNPs rs5743708, rs121917864, and rs371652530 and their associated mutation may make important functional residues changes in protein (Celniker et al., 2013).

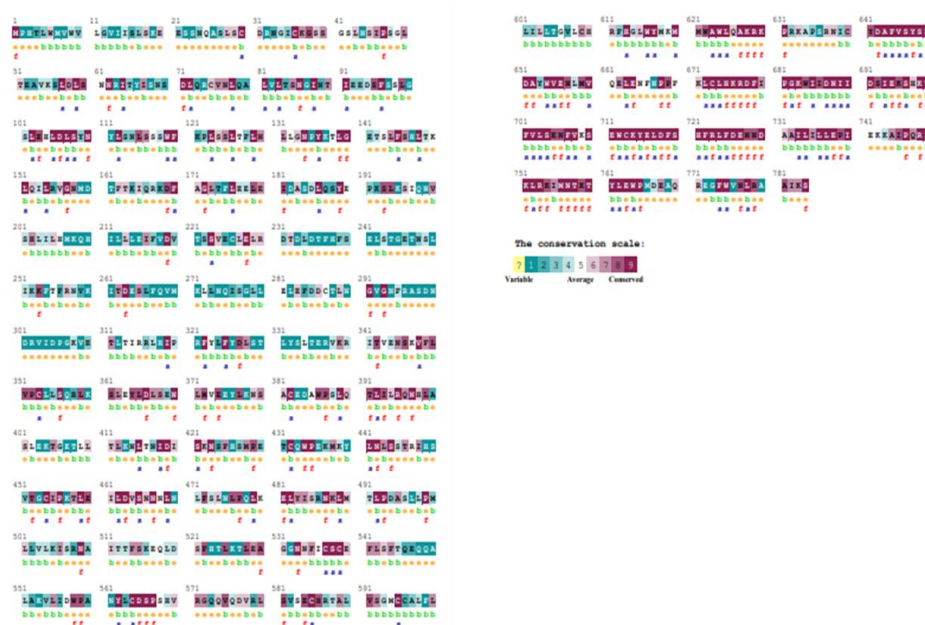


Figure 3 evolutionary conserved amino acid profile of TLR2 retrieved from ConSurf. The color-coding bar shows a conservation score

Molecular docking analysis

The interaction of the TIR domain of TLR2 (wild type and mutant type) with bacterial peptides was studied with HEPDOCK, the crystal structure of TRL2 chain A was retrieved from PDB and manually visualized by using Pymol and used for docking after removal of crystallized water molecules. About six bacterial peptides are docked with both wild type and a mutant form of TLR2. All the bacterial peptides such as MALP-2 FSL-1 (fibroblast stimulating lipopeptide-1),

LTA, peptidoglycan, porin, lipopolysaccharide, and Zymosan shows significant binding energy ranging from -143.132 Kcal/mol to -436.035 Kcal/mol (Table 3). Among the six bacterial peptides, diacylated lipopeptide FSL-1 shows good binding energy -436.035 Kcal/mol with wild type compared to other peptides. FSL-1 acts as a potent ligand of TLR2 and it was processed by macrophages after recognition. Jiménez-Dalmaroni et al. (2015) reported the binding of FSL-1f to hTLR2ED with fluorescence and native gel analysis. But it does not show much binding energy with mutant type. Followed by FSL-1, bacterial peptides glycoproteins and Zymosan showed better binding energy -184.374 Kcal/mol and -183.662 Kcal/mol respectively. Both the peptides show more or less similar binding energy with wild-type TLR2. Compared to wild type, peptidoglycan showed good binding energy in the mutant type of TLR2.

Table 3 depicted the bacterial peptides and their interacted target in innate immune response and docking score predicted by HEPDOCK.

Peptide name	Sequence	Interacted target	Docking score Wide-type TLR2	Mutant type
MALP-2	CGDPKHPKSF	TLR2	-169.125	-147.62
FSL-1	CGNNDESNISFKEK	TLR2	-436.035	-154.08
Peptidoglycan	HPWHKKHPDRKT	TLR2	-184.374	-203.15
Porin	AVDLAKIANKVLSSLF	TLR2	-143.132	-136.66
LPs	ATSEGLPILPSKVGSY	TLR2	-169.625	-145.18
Zymosan	GCGWPQHM	TLR2	-183.662	-166.00

Figure 4 represents the binding interaction of wild type and mutant type TLR2 with peptidoglycan. Compared to other peptides peptidoglycan shows higher binding affinity with both wild type and mutant type TLR2. In vitro studies have been reported that bacterial peptidoglycan is a significantly recognized TLR2 receptor and activates the cell (Yoshimura et al., 1999; Schwandner et al., 1999). Another study, the potential relevance of the SNPs in bacterial meningitis, TLR2 presents on the surface of the immune cells and effectively recognizes the cell wall components of both gram-positive and gram-negative bacteria respectively (van Well et al., 2012; Klein et al., 2008).

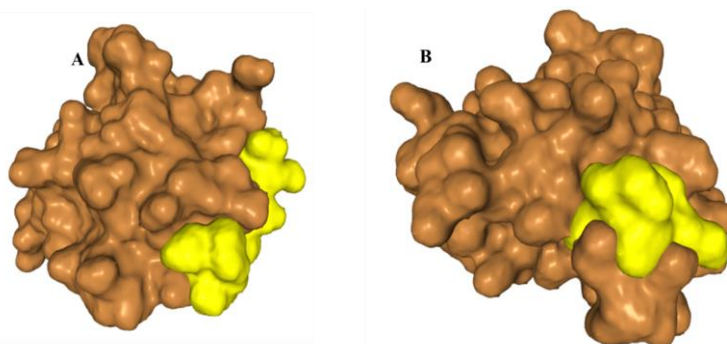


Figure 4 shows the superimposition of the docked peptide with TLR2 receptor of wild type (A) and mutant type (B). The yellow color represents the peptidoglycan and the gray color represents the TLR2 receptor

The interaction analysis of peptidoglycan with wild-type and mutant type TLR2 was examined by using PP Check web server, the mutant type TLR2 shows much docking energy, the interaction results revealed that 10 potential favorable electrostatic interactions and potential hydrogen bond interaction. The residues such as His1, His4, and Lys5 of peptidoglycan are effectively involved in the electrostatic interaction with Glu716, Asp718, and Glu727 of TLR2. The hydrogen bond formation was noticed between Lys5 of peptidoglycan with Lys714 of TLR2 via N atom of peptide and O atom of TLR2 with distance 2.04Å. Another hydrogen bond formed between His4 of peptidoglycan with Phe719 of TLR2 via Oxygen atom with the Nitrogen atom of TLR2 with a distance of 2.65Å. The important interaction residues with distance were tabulated in Tables 4 and 5. Surprisingly it did not find any potential favorable electrostatic and hydrogen bond interaction with wild type TLR2 it may be the reason for much difference in binding energy between wild type and mutant type complexes.

Table 4 represents the Potential Favorable Electrostatic Interactions between Mutant type TLR2 with peptidoglycan

RESIDUE-1 (TLR2)				RESIDUE-2 (Peptidoglycan)				Distance Å
Res. Num	Res. Name	Chain	Atom name	Res. Num	Res. Name	Chain	Atom name	
716	GLU	A	CB	1	HIS	B	CB	8.39
716	GLU	A	CB	4	HIS	B	CB	8.41
716	GLU	A	CB	5	LYS	B	CB	5.78
718	ASP	A	CB	1	HIS	B	CB	7.54
718	ASP	A	CB	4	HIS	B	CB	4.32
718	ASP	A	CB	5	LYS	B	CB	5.29
718	ASP	A	CB	6	LYS	B	CB	5.65
726	ASP	A	CB	1	HIS	B	CB	4.60
726	ASP	A	CB	4	HIS	B	CB	7.95
727	GLU	A	CB	1	HIS	B	CB	6.58

Table 5 represents the Potential hydrogen bond Interactions between Mutant type TLR2 with peptidoglycan

RESIDUE-1 (TLR2)				RESIDUE-2 (Peptidoglycan)				Type of H-bond	Distance Å
Res. Num	Res. Name	Chain	Atom name	Res. Num	Res. Name	Chain	Atom name		
714	LYS	A	O	5	LYS	B	N	BB	2.04
719	PHE	A	N	4	HIS	B	O	BB	2.65

The overall results of the current study indicate that the higher binding affinity of the six peptides with TLR2 especially the peptidoglycan shows much binding affinity with both mutant and wild type TLR2 which supports the experimental results of van Well et al. (2013). Another study carried by Zhang et al. (2017) reported the genetic variation of TLR2 in severity and prognosis of Chinese Han children associated with bacterial meningitis. The results revealed that the TLR2 SNPs are strongly associated with susceptibility to develop bacterial meningitis.

Conclusion

The Toll-like receptors are an effectively-recognized large variety of microbial pathogens and trigger the several innate immune responses that are involved with protection mechanisms and also associated with disease caused by pathogenic microorganisms. In this study, the functional and structural impact of the TLR2 gene was studied with various computational software. Out of the 6907 SNPs, 501 SNPs are present in the coding and non-synonymous region. The structural analysis of high-risk non-synonymous SNPs prediction and their associated mutation is essential for pharmacological research. The computational software used in the current study predicted the three SNPs rs5743708, rs121917864 and rs371652530 are important candidates associated with susceptibility to develop bacterial meningitis.

Competing interests

Not applicable

Funding Statement

Not applicable

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