A Bioinformatics Approach to Prioritize Single Nucleotide Polymorphisms in TLRs Signaling Pathway Genes

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Submitted 11 January 2016; Accepted 31 March 2016; Published 1 May 2016

It has been suggested that single nucleotide polymorphisms (SNPs) in genes involved in Toll-like receptors (TLRs) pathway may exhibit broad effects on function of this network and might contribute to a range of human diseases. However, the extent to which these variations affect TLR signaling is not well understood. In this study, we adopted a bioinformatics approach to predict the consequences of SNPs in TLRs network. The consequences of non-synonymous coding SNPs (nsSNPs) were predicted by SIFT, PolyPhen, PANTHER, SNPs&GO, I-Mutant, ConSurf and NetSurf tools. Structural visualization of wild type and mutant protein was performed using the project HOPE and Swiss PDB viewer. The influence of 5′-UTR and 3′-UTR SNPs were analyzed by appropriate computational approaches. Nineteen nsSNPs in TLRs pathway genes were found to have deleterious consequences as predicted by the combination of different algorithms. Moreover, our results suggested that SNPs located at UTRs of TLRs pathway genes may potentially influence binding of transcription factors or microRNAs. By applying a pathway-based bioinformatics analysis of genetic variations, we provided a prioritized list of potentially deleterious variants. These findings may facilitate the selection of proper variants for future functional and/or association studies.

Key words: Bioinformatics; in-silico analysis; single nucleotide polymorphisms; toll-like receptors

TLR-like receptors (TLRs) are a major class of the pattern-recognition receptors of the innate immune system involved in the identification of pathogen-associated molecular patterns (PAMPs) from infectious pathogens (1-2). These transmembrane proteins engage with PAMPs and trigger activation of intracellular signaling cascades, leading to the induction of genes that regulate the expression of pro-inflammatory cytokines and chemokines (3-4). Due to the critical roles of TLRs signaling network in the initiation of innate immune responses, malfunction of genes involved in this pathway may predispose individuals to numerous human diseases ranging from infectious and chronic.

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inflammatory to cancers and autoimmune diseases (5-6).

Accumulating evidence now suggests that genetic variations in TLRs pathway genes may exhibit deleterious effects on gene function, leading to the dysregulation of this signaling pathways (7-8). Single nucleotide polymorphisms (SNPs) are the shortest and the most frequent variations in the human genome. Among these, the functional consequences of untranslated regions (UTRs) and non-synonymous (nsSNPs) SNPs are of special interest, as they can either modulate gene expression or influence protein structure and function (9-10). Although the contribution of SNPs in TLR signaling to human pathological states was addressed by several studies, a comprehensive and prioritized list of SNPs potentially affecting the function and regulation of this pathway is still lacking. Therefore, this study aimed to systematically identify the UTR-SNPs and nsSNPs in genes involved in TLRs signaling network by employing a bioinformatics approach and predicting their deleterious functional and structural consequences.

Materials and methods

Retrieving SNPs in TLRs pathway genes

Data on the human TLRs pathway genes were collected from national center for biological information (http://www.ncbi.nlm.nih.gov/) (accessed May 2015) (Table 1). Genes implicated in TLRs pathway and their functional connections were retrieved by querying Kyoto encyclopedia of genes and genomes (KEGG) (http://www.genome.jp/kegg/) (accessed May 2015) (Figure 1). SNPs located in TLRs network genes were retrieved from dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) (accessed June 2015). For each SNP, the following information was recorded: SNP ID, genomic coordinate, and variation type. Protein information of TLR network genes was retrieved from UniProt (http://www.uniprot.org/) (accessed June 2015).

Predicting UTR-SNPs consequences

To evaluate the conservation score, we used genomic evolutionary rate profiling (GERP) track implemented in UCSC (https://genome.ucsc.edu/) to calculate the GERP++conservation score for each SNPs. Genomic Evolutionary Rate Profiling (GERP) is a method for producing position-specific estimates of evolutionary constraint using maximum likelihood evolutionary rate estimation. Constraint intensity at each individual alignment position is quantified in terms of a "rejected substitutions" (RS) score, defined as the number of substitutions expected under neutrality minus the number of substitutions "observed" at the position. Positive scores represent a substitution deficit (i.e., fewer substitutions than the average neutral site) and thus indicate that a site may be under the evolutionary constraint. Negative scores indicate that a site is probably evolving neutrally; negative scores should not be interpreted as evidence of accelerated rates of evolution because of too many strong confounders, such as alignment uncertainty or rate variance.

The effects of UTR-SNPs on local RNA secondary structure were predicted using mode 1 of RNAsnp program (v 1.1). The software requires RNA sequence and SNP as inputs and uses a window of 400 nucleotides, ±200 nucleotide on either side of the SNP position to obtain subsequences and generate the base-pairing probability matrix for the corresponding wild type and mutant alleles. Then, RNAsnp computes the Euclidian distance (d) and Pearson correlation coefficient (r) for all sequence intervals with a minimum length of 50 that have self-contained base pairs to assess structural difference between the wild type and mutant alleles and reports the interval with the maximum base pairing distance (dmax) or minimum correlation coefficient (rmin) along with the corresponding empirical p-value (11). Here, we used both measures independently and defined...
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structure disruptive UTR-SNPs as those with significant dmax or rmin (significance threshold is p< 0.2 as defined by RNAsnp).

RegulomeDB Version 1.1 (12) was used to annotate UTR-SNPs with known and predicted regulatory elements of the genome including the regions of DNase hypersensitivity, binding sites and motifs of transcription factors, chromatin state and the expression of quantitative trait loci.

To have further annotations, we identified 3'-UTR SNPs residing in microRNAs target sites. A comprehensive dataset of experimentally supported miRNAs target sites, including CLIP-Seq supported interactions from starBase version 2 (http://starbase.sysu.edu.cn/) (13) and CLASH verified interactions extracted from PolymiRTS database, were compiled (http://compbio.uthsc.edu/miRSNP/) (14).

| Table 1. TLR signaling pathway genes list. |
|------------------------------------------|
| **Name** | **Gene ID** | **Location** | **MIM** | **Number of SNPs** |
|---|---|---|---|---|
| 1 | TLR1 | 7096 | Chr 4 | 601194 | 321 |
| 2 | TLR2 | 7097 | Chr 4 | 603028 | 537 |
| 3 | TLR3 | 7098 | Chr 4 | 603029 | 400 |
| 4 | TLR4 | 21898 | Chr 9 | 603030 | 606 |
| 5 | TLR5 | 7100 | Chr 1 | 603031 | 790 |
| 6 | TLR6 | 10333 | Chr 4 | 605403 | 854 |
| 7 | TLR7 | 51284 | Chr X | 300365 | 544 |
| 8 | TLR8 | 51311 | Chr X | 300366 | 270 |
| 9 | TLR9 | 54106 | Chr 3 | 605474 | 509 |
| 10 | MYD88 | 4615 | Chr 3 | 602170 | 123 |
| 11 | TIRAP | 114609 | Chr 1 | 602615 | 267 |
| 12 | IRAK1 | 3654 | Chr X | 300368 | 235 |
| 13 | IRAK4 | 51135 | Chr 1 | 603029 | 601 |
| 14 | TRAF6 | 7189 | Chr 11 | 603030 | 579 |
| 15 | TRAF3 | 7187 | Chr 14 | 603031 | 2570 |
| 16 | TAB1 | 10454 | Chr 22 | 603032 | 1989 |
| 17 | TAB2 | 23118 | Chr 6 | 605101 | 3967 |
| 18 | MAP3K7 | 6885 | Chr 6 | 602614 | 1267 |
| 19 | IKBKlg | 8517 | Chr X | 300185 | 222 |
| 20 | IKBKB | 3551 | Chr 8 | 603258 | 1376 |
| 21 | CHUK | 1147 | Chr 10 | 600664 | 750 |
| 22 | NFKBIA | 4792 | Chr 14 | 164008 | 143 |
| 23 | NFKB1 | 4790 | Chr 4 | 164011 | 2060 |
| 24 | MAP2K1 | 5604 | Chr 15 | 176872 | 2124 |
| 25 | MAPK1 | 5594 | Chr 22 | 176948 | 2335 |
| 26 | MAP2K3 | 5606 | Chr 17 | 603014 | 317 |
| 27 | MAP2K7 | 5609 | Chr 19 | 600289 | 1778 |
| 28 | MAPK14 | 1432 | Chr 6 | 601158 | 2450 |
| 29 | MAPK8 | 5599 | Chr 10 | 601580 | 101 |
| 30 | FOS | 2353 | Chr 14 | 607601 | 438 |
| 31 | TICAM1 | 148022 | Chr 19 | 605048 | 1322 |
| 32 | RIPK1 | 8737 | Chr 6 | 603453 | 696 |
| 33 | IKBKE | 9641 | Chr 1 | 604834 | 895 |
| 34 | TBK1 | 29110 | Chr 12 | 603374 | 199 |
| 35 | IRF3 | 3665 | Chr 7 | 607218 | 284 |
| 36 | IRF5 | 3663 | Chr 11 | 605047 | 173 |
Fig. 1. Schematic presentation of gene network implicated in TLR signaling pathway. Direction of signal transduction is exhibited by arrows.

Analyzing the functional and structural consequences of non-synonymous SNPs

Phenotypic effects of amino acid substitution on protein function were predicted by Sorting intolerant from tolerant (SIFT) (http://sift.jcvi.org/). In this study, a list of nsSNPs (rsIDs) from NCBI's dbSNP database was submitted as a query sequence to SIFT to predict tolerated and deleterious substitutions for every position of sequence. nsSNPs with SIFT score ≤ 0.05 were classified as deleterious and those > 0.05 were classified as tolerated (15).

Polymorphism Phenotyping-2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/ pph2/) predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations. Input options for this tool are comprised of protein sequence, database ID/ accession number and details of amino acids substitution. For a given substitution, prediction outcome can be one of possibly damaging, probably damaging, and benign (16).

Protein analysis through evolutionary relationships (PANTHER) (http:// www.pantherdb. org/) estimates the likelihood of a particular nsSNPs to cause a functional impact on the protein. This tool calculates the substitution position-specific evolutionary conservation (subPSEC) score based on an alignment of evolutionarily related proteins. The subPSEC scores are continuous values from 0 (neutral) to about -10 (most likely to be deleterious). A cutoff of -3 corresponds to a 50% probability that a score is deleterious. From this, the probability that a given variant will cause a deleterious effect on protein function is estimated by P deleterious, such that a subPSEC score of -3 corresponds to a P deleterious of 0.5 (17).

SNPs database and gene ontology (GO) (http://snps.biofold.org/snps-and-go/snps-and-go.html) have been optimized to predict if a given single point protein variation can be classified as disease associated or neutral. A probability > 0.5 indicates that the mutation at the protein is disease-related (18).

ConSurf web-server (http://consurf.tau.ac.il/) is a bioinformatics tool for estimating the evolutionary conservation of amino acid positions in a protein molecule based on the phylogenetic relations between homologous sequences. The continuous conservation scores are divided into a discrete scale of nine grades for visualization, from the most variable positions (grade 1) colored turquoise, through intermediately conserved positions...
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(grade 5) colored white, to the most conserved positions (grade 9) colored maroon.

I-Mutant (http://folding.uib.es/i-mutant/i-mutant 2.0.html) is a neural network based web server for the automatic prediction of protein stability changes upon amino acid substitution. This tool provides the scores for free energy alterations, DDG<0 and DDG> 0 indicate reduction and elevation of the stability, respectively (19).

NetSurfP (http://www.cbs.dtu.dk/services/NetSurfP/) predicts the relative and absolute surface accessibility and secondary structure of residues in amino acid sequences. The reliability of the surface accessibility prediction is stated in the form of a Z-score, which cannot predict secondary structures of proteins (20).

Project Have your Protein Explained (Project HOPE) (http://www.cmbi.ru.nl/hope/home) has been used to study the insight structural features of native protein and the variant models (21). This web server provides three dimensional structural visualization of mutated proteins, and gives the results by using UniProt and DAS prediction servers.

Results

SNP analysis

Mining the dbSNP-NCBI and UniProt databases revealed a total of 35802 SNPs in thirty-seven candidate genes in TLRs pathway (Table 2). Among these, 819 and 2502 were located in 5'UTR and 3'UTR respectively, and 2172 were identified as nsSNPs.

| Categories     | Number of SNPs |
|----------------|----------------|
| Intragenic     |                |
| exon Synonymous| 1382           |
| Non-synonymous | 2172           |
| Intron         | 28654          |
| Unknown        | 273            |
| Intergenic     |                |
| 3'UTR          | 2502           |
| 5'UTR          | 819            |
| Total          | 35802          |

Fig. 2. Density plot of GERP++ conservation score (RS score). The figure shows that 5'UTR SNPs have higher (more positive) score than 3'UTR SNPs.
Conservation score of UTR SNPs

We computed GERP++scores for SNPs in UTRs, which represent an evolutionary conservation extent based on alignment of 35 mammals to hg19. Generally, 5′-UTR SNPs were found to be more conserved than 3′-UTR SNPs (Figure 2). With a cut off RS score of ≥ 2, a total of 480 constrained SNPs (including 85 5′-UTR-SNPs and 395 3′-UTR-SNPs) were identified. Moreover, 1200 SNPs (including 141 5′-UTR-SNPs and 1059 3′-UTR-SNPs) were classified as neutrally evolving, which represents a RS score of ≤0. The most conserved SNPs were found in 3′-UTR of TAB2 (rs138687718, RS score= 6.17), MAPK14 (rs377447706, RS score= 6.17) and FOS (rs45480193, RS score= 6.16).

Influence of UTR-SNPs on RNA secondary structures

Table 3. Common 3′UTR SNPs resided in miRNA target sites

| Gene  | miRNA Accession    | SNP ID     | MAF  | d_max_p-value | Conservation score |
|-------|--------------------|------------|------|---------------|--------------------|
| NFKBIA| hsa-miR-208a-3p    | rs696      | 0.46 | 0.07          |                    |
| MYD88 | hsa-miR-520f-3p    | rs7744     | 0.14 | 0.86          |                    |
| TAB2  | hsa-miR-4500      | rs7896     | 0.20 | 0.27          |                    |
| MAPK14| hsa-miR-4306      | rs8510     | 0.18 | 0.45          |                    |
| MAPK1 | hsa-miR-210-3p     | rs9340     | 0.33 | 0.21          |                    |
| MAPK1 | hsa-miR-186-5p     | rs13058    | 0.04 | 0.01          |                    |
| MAP3K7| hsa-miR-212-3p     | rs2131906  | 0.04 | 0.38          |                    |
| MAPK14| hsa-miR-381-3p     | rs3804451  | 0.13 | 0.35          |                    |
| IRAK4 | hsa-miR-340-5p     | rs4251562  | 0.04 | 0.90          |                    |
| MAP3K7| hsa-miR-212-3p     | rs9451441  | 0.01 | 0.43          |                    |
| TAB2  | hsa-miR-33a-5p     | rs35859918 | 0.01 | 0.47          |                    |
| MAPK1 | hsa-miR-217        | rs41282607 | 0.01 | 0.08          |                    |
| TAB2  | hsa-miR-539-5p     | rs41288431 | 0.01 | 0.82          |                    |
| MAPK1 | hsa-miR-488-3p     | rs61757976 | 0.01 | 0.76          |                    |
| TRAF3 | hsa-miR-4500      | rs72704737 | 0.29 | 0.12          |                    |

1 Target miRNA SNP MAF d_max p-value
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Our analysis showed that 313 UTR-SNPs were structure disruptive as defined by dmax p-value P<0.2 (Figure 3). Considering both dmax and rmin, there were 232 unique structure disruptive UTR-SNPs. The top five genes enriched for structure disruptive SNPs were MAPK14 (n= 23), TLR7 (n= 12), TLR4 (n= 10), MAPK1 (n= 10), and TRAF3 (n= 8).

Annotation of SNPs with regulatory elements

Disease associated variants are enriched in regulatory elements of the genome. Using RegulomeDB, we annotated UTR-SNPs within regulatory elements. 11 UTR-SNPs were associated with transcription factor binding sites (i.e eQTL).

Identification of SNPs residing in miRNA target sites

Intersecting 3’UTR-SNPs with the experimentally validated miRNAs target site datasets, we found 314 SNPs resided in microRNAs target sites. Since miRNA target sites are under selective pressure, we refined SNPs in miRNA target sites by minor allele frequency (MAF) threshold of 0.01 (Table 3).

Table 4. List of nsSNPs that predicted to be deleterious by both PolyPhen-2 and SIFT tools

| Gene Symbol | SNP     | Allele | AA substitution | PolyPhen Score | PolyPhen Prediction | SIFT Score | SIFT Prediction |
|-------------|---------|--------|-----------------|----------------|---------------------|------------|-----------------|
| 1 CHUK      | rs56948661 | G>A    | P623L          | 1              | P.D                 | 0.01       | Damaging        |
| 2 CHUK      | rs61732515 | C>G    | Q277H          | 0.999          | P.D                 | 0.00       | Damaging        |
| 3 CHUK      | rs112432667| T>C    | E492G          | 0.954          | P.D                 | 0.00       | Damaging        |
| 4 FOS       | rs74685695 | T>G    | V77G           | 0.999          | P.D                 | 0.01       | Damaging        |
| 5 TIRAP     | rs112815033| T>C    | L450P          | 1              | P.D                 | 0.01       | Damaging        |
| 6 IRAK4     | rs55944915 | G>A    | R391H          | 0.999          | P.D                 | 0.01       | Damaging        |
| 7 IRAK4     | rs114820168| C>T    | R391C          | 1              | P.D                 | 0.00       | Damaging        |
| 8 MAP3K7    | rs77759048 | A>T    | W55R           | 1              | P.D                 | 0.00       | Damaging        |
| 9 TBK1      | rs34774243 | A>G    | K291E          | 0.997          | P.D                 | 0.00       | Damaging        |
| 10 TBK1     | rs55824172 | C>T    | S151F          | 0.997          | P.D                 | 0.00       | Damaging        |
| 11 TIRAP    | rs74937157 | T>C    | C134R          | 1              | P.D                 | 0.00       | Damaging        |
| 12 TLR1     | rs5743621 | G>A    | P733L          | 0.995          | P.D                 | 0.00       | Damaging        |
| 13 TLR1     | rs41311402 | A>G    | L697S          | 1              | P.D                 | 0.00       | Damaging        |
| 14 TLR1     | rs56205407 | A>G    | I679T          | 0.999          | P.D                 | 0.00       | Damaging        |
| 15 TLR1     | rs117033348| A>G    | L144P          | 1              | P.D                 | 0.04       | Damaging        |
| 16 TLR2     | rs5743706 | T>A    | Y715N          | 1              | P.D                 | 0.01       | Damaging        |
| 17 TLR2     | rs56303479 | T>C    | L81P           | 1              | P.D                 | 0.00       | Damaging        |
| 18 TLR2     | rs121917864| C>T    | R677W          | 1              | P.D                 | 0.00       | Damaging        |
| 19 TLR3     | rs5743316 | A>T    | N284I          | 1              | P.D                 | 0.00       | Damaging        |
| 20 TLR3     | rs112666555| T>C    | L545P          | 1              | P.D                 | 0.00       | Damaging        |
| 21 TLR3     | rs111488413| C>A    | P880Q          | 1              | P.D                 | 0.00       | Damaging        |
| 22 TLR4     | rs77214890 | G>T    | D181Y          | 1              | P.D                 | 0.00       | Damaging        |
| 23 TLR4     | rs80197996 | G>T    | L470F          | 1              | P.D                 | 0.03       | Damaging        |
| 24 TLR4     | rs55905951 | C>G    | A676G          | 1              | P.D                 | 0.00       | Damaging        |
| 25 TLR4     | rs55786277 | C>T    | R804W          | 0.999          | P.D                 | 0.01       | Damaging        |
| 26 TLR5     | rs5744176 | T>C    | D694G          | 1              | P.D                 | 0.01       | Damaging        |
| 27 TLR5     | rs78098893 | T>C    | R752G          | 0.997          | P.D                 | 0.01       | Damaging        |
| 28 TLR6     | rs13102250 | A>C    | L105W          | 1              | P.D                 | 0.01       | Damaging        |
| 29 TLR9     | rs55881257 | G>A    | R962C          | 1              | P.D                 | 0.01       | Damaging        |

Abbreviations: P.D; probably damaging
Prediction of tolerated and deleterious non-synonymous SNPs by SIFT
SIFT analysis predicted that a total of 785 nsSNPs were damaging (score< 0.05) and 1322 nsSNPs had tolerated effects on the candidate genes involved in TLR pathway network (score> 0.05) (Figure 4).

Prediction of damaging non-synonymous SNPs by PolyPhen-2
According to our Polyphen-2 results, 610 nsSNPs were predicted “probably damaging”, 353 nsSNPs were predicted “possibly damaging” and 1068 were classified as benign (Figure 4). To increase the accuracy of predictions, results of SIFT and PolyPhen-2 were joined and SNPs with PolyPhen score> 0.95 and SIFT< 0.05 were selected. Accordingly, 29 nsSNPs passed both criteria and were classified as deleterious/damaging (Table 4).

Prediction of functional impact of non-synonymous SNPs on protein by PANTHER and SNPs & GO.
According to the PANTHER results, all 29 SNPs possessed the subPSEC score more than −3 and were therefore classified as deleterious (Table 5). As shown in table 5, these SNPs were found to be as disease-associated with the probability >0.5 after analyzing by SNPs & GO.

Prediction of protein stability analysis by I-Mutant
According to I-Mutant results, all mutations expect N284I (rs5743316 in TLR3), S151F (rs55824172 in TBK1) and L105W (rs13102250 in TLR6) were predicted to decrease protein stability, with a free energy change value ≤0.0 (Table 6).

Prediction of evolutionary conservation of amino acid position by ConSurf
Our ConSurf analysis revealed that all 29 expected SNPs including the Q277H (CHUK), E492G (CHUK), L450P (IRF5), W55R (MAP3K7), K291E (TBK1), C134R (TIRAP), I679T (TLR1), L545P (TLR3), R804W (TLR4) and R752G (TLR5) were located in highly conserved regions and predicted to have functional and structural impacts on TLRs pathway proteins (Table 6).
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type and mutant protein containing the mentioned deleterious variants was performed using the project HOPE and Swiss PDB viewer.

**Table 5. PANTHER and SNPs&GO results for prediction of SNPs as disease associated.**

| SNPs     | Substitution | PANTHER subPSEC | Pdeleterious | Prediction | RI | Probability |
|----------|--------------|-----------------|--------------|------------|----|-------------|
| rs56948661 | P623L        | -4.92855        | 0.87309      | Disease    | 5  | 0.742       |
| rs61732515 | Q277H        | -4.61589        | 0.83423      | Disease    | 3  | 0.527       |
| rs112432667 | E492G       | -3.99182        | 0.72945      | Disease    | 4  | 0.711       |
| rs74685695  | V77G         | -4.06862        | 0.74433      | Disease    | 1  | 0.545       |
| rs112815033 | L450P        | -4.36601        | 0.79674      | Disease    | 0  | 0.523       |
| rs55944915  | R391H        | -3.64924        | 0.65684      | Disease    | 0  | 0.525       |
| rs114820168 | R391C        | -4.67097        | 0.84171      | Disease    | 3  | 0.643       |
| rs77759048  | W55R         | -3.3007         | 0.57461      | Disease    | 4  | 0.717       |
| rs34774243  | K291E        | -3.56533        | 0.63768      | Disease    | 5  | 0.772       |
| rs55824172  | S151F        | -4.7119         | 0.84708      | Disease    | 6  | 0.804       |
| rs74937157  | C134R        | -3.47178        | 0.6158       | Disease    | 2  | 0.619       |
| rs5743621   | P733L        | -4.51666        | 0.82005      | Disease    | 2  | 0.623       |
| rs41311402  | L697S        | -4.23845        | 0.77529      | Disease    | 4  | 0.712       |
| rs56205407  | I679T        | -5.35855        | 0.91361      | Disease    | 7  | 0.870       |
| rs117033348 | L144P        | -8.17834        | 0.99439      | Disease    | 5  | 0.750       |
| rs5743706   | Y715N        | -4.34331        | 0.79303      | Disease    | 4  | 0.707       |
| rs56303479  | L81P         | -6.4936         | 0.97051      | Disease    | 7  | 0.855       |
| rs121917864 | R677W        | -6.4688         | 0.96979      | Disease    | 6  | 0.819       |
| rs5743316   | N284I        | -3.91448        | 0.71392      | Disease    | 5  | 0.748       |
| rs112666655 | L545P        | -4.25641        | 0.77841      | Disease    | 6  | 0.823       |
| rs111488413 | P880Q        | -8.50881        | 0.99597      | Disease    | 6  | 0.811       |
| rs77214890  | D181Y        | -4.48068        | 0.81467      | Disease    | 0  | 0.511       |
| rs80197996  | L470F        | -3.94106        | 0.71931      | Disease    | 4  | 0.639       |
| rs55905951  | A676G        | -3.16208        | 0.54043      | Disease    | 0  | 0.503       |
| rs55786277  | R804W        | -5.10263        | 0.89116      | Disease    | 5  | 0.748       |
| rs5744176   | D694G        | -3.42967        | 0.6058       | Disease    | 4  | 0.716       |
| rs78098893  | R752G        | -3.16919        | 0.5422       | Disease    | 2  | 0.614       |
| rs13102250  | L105W        | -5.09383        | 0.8903       | Disease    | 2  | 0.583       |
| rs55881257  | R962C        | -4.48094        | 0.81471      | Disease    | 1  | 0.547       |

**Table 5. PANTHER and SNPs&GO results for prediction of SNPs as disease associated.**

these mutations were analyzed for solvent accessibility and stability, and the results were represented in the following paragraphs (see also Table 7). Visualization of structural features of wild
The rs56948661 in CHUK gene leads to P623L. The residue is located on the surface of the protein and mutation of this residue can disturb the interactions with other molecules or other parts of the protein. Moreover, the mutation can disturb the special backbone conformation induced by proline. Conversion of V77G (rs74685695 in FOS) causes some structural changes in protein. Glycine residue is smaller than valine and this may lead to loss of the interactions. Furthermore, the mutant residue is more hydrophobic and flexible and can disturb the required rigidity of the protein on this position. For rs114820168 in IRAK4, the wild-type (arginine) and mutant (cysteine) amino acids differ in size,
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hydrophobicity and charge. The difference in charge will disturb the ionic interactions of the wild type residue with D388, E389 and D398. R391H is annotated with rs55944915 in dbSNP database. According to the PISA-database, the mutated residue is involved in a multimer contact. The new residue might be too small to make multimer contacts. In S151F variant, rs55824172 of TBK1 gene, the mutant residue (phenylalanine) is bigger and more hydrophobic than the wild-type (serine). This conversion will cause the loss of hydrogen bonds in the core of the protein resulting in the disruption of correct folding.

We found that three SNPs in TLR1, including P733L (rs5743621), L697S (rs41311402) and L144P (rs117033348), were located in highly conserved regions and predicted to have functional and structural impacts on proteins. For P733L, the mutant residue (leucine) is bigger than the wild-type (proline) and is located on surface of the protein, potentially disturbing its interactions. For L697S and L144P, the mutant residues are smaller than the wild-type residues and will cause an empty space in the core of the protein. In addition, all three mutations are predicted to have functional and structural influences on TLR2 protein (Figure 5).
For L81P (rs56303479), because this residue is part of some interpro domains like leucine-rich repeat, typical subtype, the interaction between these domains could be disturbed by the mutation. The R677W (rs121917864) mutation leads to substitution of arginine by a bigger and more hydrophobic residue named tryptophan. The difference in charge will disturb the ionic interaction made by the arginine with E649 and 656. The third mutation of TLR2 occurs at position 715 (rs5743706). The hydrophobicity of the wild-type (tyrosine) and mutant residue (asparagine) differs and the mutation will cause the loss of hydrophobic interactions in the core of the protein. Finally, the size difference between residues makes that the new residue is not in the correct position to make the same hydrogen bond with S646, as the wild-type residue does. For N284I (rs5743316, in int.
TLR3), due to the difference in hydrophobicity index of residues, the mutation will cause the loss of hydrogen bonds in the core of the protein and may lead to incorrect folding of protein. The second mutation of TLR3 (rs111488413) causes P880Q. This mutant residue is bigger than the wild-type residue and can disturb the protein interactions. Additionally, the hydrophobicity of the residue differs; hence, the mutation may cause the loss of hydrophobic interactions.

Concerning D181Y mutation in TLR4 (rs77214890), the difference in charge will disturb the ionic interaction made by the original residue with R234. Moreover, the hydrophobicity of the native and mutant residue differs. Therefore, this mutation causes the loss of hydrogen bonds in the core of the protein leading to disruption of the correct folding (Figure 6). For rs80197996 (L470F) in TLR4, the mutant residue (phenylalanine) is bigger and probably will not fit to bury in the core of the protein. In A676G (rs55905951), the mutant residue is smaller than the wild-type residue. This will cause a possible loss of external interactions. Furthermore, the mutation may cause the loss of hydrophobic interactions with other molecules on the surface of the protein.

Concerning rs5744176 (D694G) of TLR5, the wild-type residue forms a salt bridge with K692, R752 and K753. The difference in charge will disturb these ionic interactions. Moreover, the aspartic acid forms a hydrogen bond with N726, but due to difference in hydrophobicity, the mutation causes the loss of hydrogen bond. For the L105W (rs13102250) in TLR6, the wild-type (leucine) and mutant (tryptophan) amino acids differ in size. The wild-type residue was buried in the core of the protein, but the mutant residue is bigger and probably will not fit. For rs55881257 (R962C in TLR9) the charge of the wild-type residue will be lost; this can cause the loss of interactions with other molecules or residues. Furthermore, this mutation introduces a more hydrophobic residue at this position, probably resulting to loss of hydrogen bonds.

Discussion

TLRs signaling pathway plays a key role in the host innate immune response. Increasing evidence has suggested that functional SNPs of genes related to TLRs pathway may contribute to diseases ranging from chronic inflammatory to cancers. Since SNPs are the most common genetic variations in human genome, it is expected that genes involved in TLRs pathway contains numerous SNPs. Nevertheless, discriminating deleterious SNPs with potential effects on disease susceptibility from tolerated variants is a major challenge. Therefore, a comprehensive study that systematically analyzes the effects of such SNPs can cost-effectively prioritized SNPs for further analyzes.

In-silico analysis of the deleterious effects of SNPs may help to improve our understanding on the biological pathways (22). In this study, we systematically analyzed the SNPs in different parts of genes (5′-UTR, 3′-UTR and coding) in TLRs pathway. A report has suggested that mutation effect prediction algorithms have their own strengths and weaknesses, and therefore, implementing a combination of these tools may help to enhance the accuracy of effect predictions (23). In the present study, we combined the results of the SIFT, PolyPhen, PANTHER, SNPs & GO, I-Mutant and ConSurf algorithms to prioritize the damaging nsSNPs and increase the analysis accuracy. Accordingly, we were able to identify several potentially deleterious nsSNPs in TLRs pathway genes. These SNPs, to the best of our knowledge, have not yet been investigated and therefore may be considered as candidates for association with diseases. These results may pave the ground for future functional and/or association
studies and facilitate the process of choosing functional variant for further analyses.

UTR-SNPs play important roles in gene regulation and accumulating evidence has indicated their contribution to different diseases. Sequence alteration in these regulatory elements has been shown to interfere with transcription factors or microRNA binding, leading to gene dysregulation (24-25). By applying a bioinformatics approach, we evaluated such effects of UTR-SNPs on TLRs pathway genes and identified numerous disease-associated variants that potentially confer the disease risk through affecting transcription factors or miRNAs binding. TLR9 rs187084, a UTR-SNP which probably interferes with transcription factors binding, has been shown to modify susceptibility to diseases specially renal transplant recipients and cancers (26-27). Several genes of TLRs pathway are regulated post-transcriptionally by miRNAs (28). Our analysis revealed that several SNPs of TLRs network resided in microRNA target sites (Table 3) that may potentially modify miRNA-mediated regulation of these genes. For instance, rs7744 in 3′-UTR of MYD88 and rs696 in 3′-UTR of NFKBIA genes could disrupt the binding of miR-520f-3p and miR-208a-3p, respectively. Matsunaga et al. showed that homozygous minor allele of rs7744 is associated with the severity of ulcerative colitis (29). Moreover, it has been shown that rs696 G>A is associated with the susceptibility to different diseases including coronary artery disease and Behçet’s disease (30-31).

In conclusion, the current study reports the first pathway-based bioinformatics analysis of SNPs in TLRs pathway genes and provides a prioritized list of functional SNPs potentially affecting regulation and function of the pathway. However, we noticed that the complexities of biological pathways merit the need for more experimentation to validate the true effect of these SNPs on TLRs network. Although the functional significance of the candidate SNPs was not experimentally assessed in this study, we believe that our results will help researchers interested in the roles of SNPs in TLRs pathways genes to focus on proper candidate variants.

Acknowledgements

We would like to thank the Deputy of Research of Tehran University of Medical Sciences for the financial support of this study project. Grant No: 93-02-30-25172.

Conflict of Interests

The authors declared no conflict of interests.

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