Screening for Deleterious non-synonymous SNPs in Human CCL21 Gene using in-silico analysis

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Abstract

Rheumatoid arthritis (RA) is a chronic, systematic, and progressive inflammatory disorder, causing severe damage to joints and hence increase mortality. The Chemokine (C-C motif) ligand 21 (CCL21), a member cytokines family, is involved in immuno-inflammatory and regulatory processes. Therefore, identifying the important SNPs (single nucleotide polymorphisms) in the CCL21 gene is of key importance to evaluate their structural and functional significance and to discover novel therapeutic targets for immune-related diseases, including RA. In this study, we used in silico approaches for identifying the most damaging non-synonymous SNPs (nsSNPs), playing a significant structural and functional role in CCL21 protein. The primary tools used for this study included PROVEAN, SNPs&GO, SIFT and PolyPhen2. Other tools, its stability, Structure and functional effect as well as the conservation profile, were verified using I-Mutant, MutPred, and ConSurf. The site of post-translational modification also predicted. The 3-D modeling of proteins was carried out using I-TASSER which were then visualized in Chimera v1.11. Furthermore, the gene-gene interactions were predicted using STRING and gene MANIA.

It was observed that the nsSNPs D30Y (rs753133670), I62N (rs1170851787), R75C
(rs759733358), R75S (rs776954599) and A83V (rs776954599) were the most damaging nsSNPs in the CCL21 gene. These nsSNPs might have a significant role in CCL2 protein’s malfunctioning and possibly causing different autoimmune diseases including RA. Our study concluded that, to study the correlation of the CCL21 gene with certain autoimmune disorders, i.e. Crohn’s Disease (CD), RA and other immune-associated diseases, these SNPs could be the most important ones. In addition, these SNPs need to be studied in animal models and cell cultures in association with certain diseases, to identify if they could be of use for the gene therapy and pharmacogenomics.

**Keywords**: CCL21; nsSNPs; Polymorphisms; Gene-gene interactions; In silico.

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**Introduction**
Rheumatoid arthritis (RA) leads to inflammation in joints and articular cartilage coupled with synovial hyperplasia, thereby characterized as an autoimmune disease, and causes consistent pain and permanent disability of the patients’ physical activities in normal life [1, 2]. The pathogenesis of RA is still unclear but the incidence and prevalence of RA are said to be the result of different environmental and genetic risk factors [3]. On the basis of proposed data by different studies (family aggregation and twin consonance), RA was found heritable in 60% of patients. These variations indicate the role of genetic factors in the pathogenesis of RA [4, 5]. The roles of SNPs have been detected in the non-MHC genes, such as PTPN22 and MHC genes like HLA-DRB1 which are potent and can drive inflammatory response in RA. Many studies revealed >150 SNPs in RA located at more than 70 gene loci [6-10].

*CCL21* gene belongs to the Chemokine family having a C-C motif and is located on chromosome 9p13.3. *CCL21* is the
chemokine that binds to CCR7 and plays an important role by modulating the process of circulation in the lymphoid and peripheral organs of T cells as well as dendritic cells [11]. In addition, defective movement of dendritic cells and lymphocytes into T zones has been demonstrated in CCR7 deficient mice [12]. Previous studies have shown that the endothelial cell growth factor CCL21 is mediated/expressed through the endothelial cell lymph node, which is related to tertiary lymphoid tissue development [13, 14]. Recent research on RA patients revealed an elevated expression of both the CCR7 and CCL21 in fibroblasts and macrophages of endothelial sub-lining cells and synovial tissues. Macrophages in synovial fluids of RA patients produce a high number of CCL21 than normal in vitro differentiated peripheral blood macrophages [15, 16].

In RA pathogenesis, the up-regulation of the CCL21 gene in synovial tissue linings is dependent on the activation of the fibroblast and macrophages, which in turn produces proangiogenic factors, i.e. vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), and Angiogenin1. The high expression of CCL21 gene has been observed in the sub-lining of endothelial cells than the peripheral ordinary blood cells [17].

Currently, research on RA-associated polymorphisms in CCL21 gene is not only rare but also has diverse outcomes. These differences can be attributed to the small sample sizes and variations in genetic make-up [18]. Many studies have reported that nsSNPs in various genes like TAGAP, TOX3 and CCR6 may negatively impact protein structure and function and might be correlated to diseases like Breast cancer, RA, and others [19-22].

2. METHODOLOGY

2.1 Recruiting nsSNPs
Data regarding the location, global minor allele frequencies (MAFs) and residual changes of SNPs in the CCL21 gene were taken from NCBI dbSNP [23]. After analyzing the data, 916 SNPs were searched, out of them, 96 nsSNPs were selected for further processing.

2.2 Finding the most damaging CCL21 nsSNPs
Four bioinformatics tools, including (a) SIFT (Sorting Intolerant from Tolerant) [24], (b) PROVEAN (Protein Variation Effect Analyzer) [25], (c) SNPs& [26] and (d) PolyPhen2 (Polymorphism Phenotyping2) were applied to identify the deleterious
nsSNPs [27]. Further screening was done on the nsSNPs predicted by all the tools as likely deleterious or intolerant.

2.3 Effect of nsSNPs in CCL21 protein

To predict the structural as well as functional effects of the given deleterious nsSNPs on the protein product MutPred tool was used [28]. An online web-based MutPred tool identifies alteration in the sequence of amino acid and predicts the root cause of the disease as well. This tool is mostly used for screening the physical and functional characteristics like phosphorylation site loss or propensity gains. Deleterious nsSNPs were selected from the FASTA sequence of the CCL21 gene with p < 0.05 being deliberated as assured and p <0.01 being considered as very assured.

The I-Mutant 2.0, a web based tool was applied to test the stability of protein. It shows stability variations in the altered protein and gives us predictions about RI starting from 0 to 10. Where 0 is the lowest and 10 is the highest possible RI values [29]. Further analysis was carried out on those nsSNPs which were found to reduce CCL21 protein stability. Each amino acid was identified by the use of ConSurf tool) based on evolutionary conservation. It also illustrates the phylogenetic relationships between homologous sequences [30]. Further analysis was performed only for those sequences which were found highly conserved and which also showed more similarity with lethal nsSNPs.

2.4 Protein Modeling

In this study, I-TASSER was to modeled 3D structures of wild type and mutant CCL21 [31]. We used Chimera 1.11 to visualize the resultant protein structures and study molecular features. TM-align was used to analyze the structural differences, when the wild-type CCL21 protein was compared to the mutant CCL21 proteins. TM-align calculates root mean square deviation (RMSD), structural superposition and template modelling (TM) score. The structural similarity was predicted by TM score that ranges from 0 to 1, where 1 shows higher resemblance. The variation depends upon the RMSD value, greater the RMSD value, higher will be the variation between wild and mutant structures [32].

2.5 Prediction of possible Post Translational Modification (PTM) Sites

Different tools were applied for identification of possible PTM sites in CCL21 protein. GPS-MSP 3.0, an online tool, was applied to identify the methylation sites in CCL21 protein [33] whereas, possible sites for phosphorylation in the protein were anticipated through NetPhos 3.1 and GPS.
3.0. The phosphorylation sites were predicted at the threonine, tyrosine and serine positions of the CCL21 protein. NetPhos 3.1 predicted less specific results having a lower phosphorylation potential than GPS 3.1 [34]. In addition, ubiquitylation sites were predicted with the help of BDM-PUB and UbPred) tools [35].

2.6 Gene-gene Interaction and effect of regulatory region SNPs
The interaction and association of CCL21 with other proteins and its nsSNPs’ effects on other proteins were studied by utilizing two in silico tools i.e., GeneMANIA and STRING [36, 37]. The GeneMANIA finds out the interaction between genes on the basis of protein interaction, protein domain similarity, co-expression, co-localization and pathways. STRING calculations are based on top 10 strongly interacting proteins for which the limitation includes co-expression, gene fusion, co-occurrence, biochemical and experimental data. It predicts collective score of each protein, which interacts with the target gene and ranges from 0 to 1, where 1 and 0 are the highest and lowest interactions, respectively. CCL21 gene was submitted and interactions between genes were predicted. MicroSNiPer and PolymiRTS Database were used to study whether these nsSNPs in CCL21 gene has a role in gene regulation [38]. MicroSNiPer particularly show whether or not the target SNPs containing region would impact miRNA. PolymiRTS database, which is a web based server ensuring that variants in the UTR regions as well as in miRNA seed are affected by deleterious SNPs.

3. RESULTS
3.1 nsSNPs Recruitment
CCL21 gene has 916 SNPs; 96 and 23 are located in the 5'UTR and in the 3'UTR, respectively. In addition, other types of SNPs identified as splice sites, intronic, synonymous, and uncategorized. (Figure 1). Of the 96 nsSNPs selected for further analysis, 7 nsSNPs resulted in stop codons, thus affecting the protein structure directly. Seven of these nsSNPs can result in truncated protein that can potentially lead to disease.
3.2 Characterization of Deleterious nsSNPs in CCL21 Gene

Four bioinformatics tools (PROVEAN, SIFT, SNPs&GO and polyPhen-2) were used in the analysis of 96 nsSNPs recruited from dbSNPs to identify the possible hazardous structural and functional outcomes of these nsSNPs on CCL21 protein. According to the PROVEAN, 25 nsSNPs were predicted deleterious effects while SIFT predicted total 39 nsSNPs as intolerable. Moreover, the SNPs&GO tool evaluated 37 nsSNPs in total as disease causing. Of these, 12 nsSNPs were declared damaging, to the final protein, by all three tools. These shortlisted nsSNPs were later subjected to PolyPhen2, which display its results in three categories i.e. benign, possibly and probably damaging. It predicted 7 nsSNPs as potentially damaging. It also gives a count between 0 and 1 where 1 is considered as the most damaging hence all further investigations were carried out for 7 nsSNPs having score 1 (Table 1).

| SNP ID | Amino acid position of SNP | Provean Score | SNP &Go Probability | SIFT RI Score | Polyphen-2 Score |
|--------|---------------------------|---------------|---------------------|---------------|-----------------|
| rs779706400  | L7P   | -4.530 | 0.902 | 8  | 0.04 | 1  |
| rs753133670  | D30Y  | -7.122 | 0.917 | 8  | 0.02 | 1  |
| rs1453433779 | R46C  | -3.858 | 0.843 | 7  | 0.01 | 1  |
| rs1170851787 | I62N  | -5.576 | 0.733 | 5  | 0.01 | 0.999 |
| rs759733358  | C75R  | -11.422 | 0.998 | 10 | 0.02 | 1  |
| rs776954599  | C75S  | -9.736 | 0.997 | 10 | 0.02 | 1  |
| rs1182863895 | V83A  | -3.911 | 0.977 | 10 | 0.00 | 0.999 |

**Figure 2** Percent prediction of nsSNPs by SIFT, Provean, SNP&GO and polypen-2

### 3.3 Effect of nsSNPs in CCL21 protein

The damaging effect of the finalized nsSNPs on the structure or function of CCL21 protein was predicted through the MutPred server. The results are given in Table 2. I-Mutant predicted the influence of the selected 7 nsSNPs on CCL21 protein stability. Each nsSNP was submitted separately for RI
calculations (ranging from 0 to 10) to determine whether stability should be decreased/increased results are given in Table 3. Out of 7 shortlisted nsSNPs, 2 were shown to increase the protein’s stability i.e., substitution with L7P (rs779706400) and R46C (rs1453433779) and hence were omitted for further study whereas rest of the 5 nsSNPs showed a decrease in stability of CCL21 protein (Table 3). These 5 nsSNPs were chosen for further investigation. The conservation profile of these nsSNPs was predicted through the ConSurf tool which depicted C57R, C75S and V83A as highly conserved, exposed structural residues. The amino acid D30 was predicted to be highly conserved, exposed and functional residue, while the amino acid I62 was predicted to be buried. Retention scores for all the selected nsSNPs are depicted in Table 4. The results indicated that the nsSNPs with high conservation were most damaging for structure and function of CCL21 protein.
Table 2: Effects of nsSNPs on structural & functional properties of CCL21 by MutPred server

| Mutation | Probability of deleterious mutation | Top Features | Affected PROSITE and ELM Motifs |
|----------|------------------------------------|--------------|---------------------------------|
| L7P      | 0.862                              | Altered Signal peptide (p=1.5e-05) | ELME000053                |
|          |                                    | Altered Stability (p=0.04)            | ELME000248                |
| D30Y     | 0.839                              | Altered Transmembrane protein (P = 2.1e-04) | ELME000120 |
|          |                                    | Loss of Disulfide linkage at C32 (P = 1.2e-03) |                  |
|          |                                    | Loss of Allosteric Site at Y84 (P = 0.04) | ELME000182 |
|          |                                    | Loss of catalytic site at C32 (P = 0.04) |                  |
|          |                                    | Gain of Pyrrolidone carboxylic acid at Q29(P=0.02) |                  |
|          |                                    | Altered signal peptide (P = 4.3e.03) |                  |
| R46C     | 0.219                              | -                                        | None                |
| I62N     | 0.894                              | Altered Stability (P=1.6e-03)            | ELME000276 |
|          |                                    | Altered Transmembrane protein (p=2.6e-04) |                  |
|          |                                    | Altered Ordered interface (p=0.03)       |                  |
|          |                                    | Gain of Disulfide linkage at C57 (p=9.9e-04) |                  |
| C75R     | 0.950                              | Altered Metal binding (p=1.7e-03)         | None            |
|          |                                    | Loss of Disulfide linkage at C57 (p=4.1e-04) |                  |
|          |                                    | Altered Disordered interface (p=0.01)     |                  |
|          |                                    | Gain Of intrinsic (p=0.02)                |                  |
|          |                                    | Gain of Catalytic site at C75 (p=0.4e-03) |                  |
|          |                                    | Altered Transmembrane protein (p=0.01)     |                  |
|          |                                    | Altered Transmembrane protein (p=0.02)     |                  |
| C75S     | 0.906                              | Altered Metal binding (p=1.3e-03)         | ELME000085 |
|          |                                    | Loss of Disulfide linkage at C57 (p=4.1e-04) |                  |
|          |                                    | Altered Disordered interface (p=0.02)     | ELME000147 |
|          |                                    | Gain of Catalytic site at C75 (p=0.01)     |                  |
|          |                                    | Altered transmembrane protein (p=0.01)     |                  |
| V83A | 0.564 | Gain of intrinsic disorder (p=0.417.2e-03) |
|------|-------|-----------------------------------------|
|      |       | Altered Disordered interface (p=0.320.02) |
|      |       | Altered transmembrane protein (p=0.01)   |
|      |       | Altered Coiled coil (P=0.04)             |
|      |       | ELME000106                                |
Table. 3 I-Mutant Results for the nsSNPs

| SNP ID       | SNP position | Stability | RI | SNP ID       | Amino acid Change | Stability  | RI |
|--------------|--------------|-----------|----|--------------|-------------------|------------|----|
| rs779706400  | L7P          | Increased |    | s759733358   | C75R              | Decreased  |    |
| rs753133670  | D30Y         | Decreased |    | rs776954599  | C75S              | Decreased  |    |
| rs1453433779 | R46C         | Increased |    | rs1182863895 | V83A              | Decreased  |    |
| rs1170851787 | I62N         | Decreased |    |              |                   |            |    |

Table. 4 Conservation profiling of the target amino acids, where the selected nsSNPs are located

| SNP ID       | Amino acid positions | Conservation Score | Prediction                           |
|--------------|-----------------------|--------------------|--------------------------------------|
| rs753133670  | D30                   | 8                  | Exposed and Highly Conserved (f)     |
| rs1170851787 | I62                   | 8                  | Buried                               |
| s759733358   | C75                   | 9                  | Exposed and Highly Conserved (s)     |
| rs776954599  | C75                   | 9                  | Exposed and Highly Conserved (s)     |
| rs1182863895 | V83                   | 9                  | Buried and Highly Conserved (s)      |

3.3 CCL21 and its Mutants protein 3D-modelling

The 5 nsSNPs which were found to be decreasing the stability of CCL21 protein were further selected for protein modeling. CCL21 proteins PDB file was generated by I-TASSER using the protein sequences of CCL21 protein (wild-type and mutants). I-TASSER used templates 2co9 and 2nbiA (83% identity, 85% coverage of thread alignment). The PDB files for each mutant model along with their TM-scores and RMSD values are given in Table 5. Besides, the structural and molecular characterizations of the protein were analyzed using Chimera 1.11 (Figure2).
Table 5: RMSD values and TM score of 5 selected nsSNPs

| SNP ID      | Residual Change | TM Score | RMSD Values | SNP ID      | Residual Change | TM Score | RMSD Values |
|-------------|-----------------|----------|-------------|-------------|-----------------|----------|-------------|
| rs753133670 | D30Y            | 0.79644  | 2.9         | rs776954599 | C75S            | 0.82035  | 2.51        |
| rs1170851787| I62N            | 0.78680  | 2.81        | rs1182863895| V83A            | 0.83601  | 2.22        |
| rs759733358 | C75R            | 0.8898   | 2.38        |             |                 |          |             |

(A) Wild type CCL21 protein

(B) Tyrosine (Mutant)
Aspartic acid (wild)
Figure 3 A). Protein structure of CCL21 wild type, B) Superimposed Structure of D30Y mutant, C) I62N mutant, D) C75S mutant, E) C75SR mutant, and F) V83A mutant.
3.4 Predicted Post Translational Modifications Site (PTMs)

The structures and functions of proteins are controlled by PTMs, the key events in biological systems such as cell signaling and protein-protein interactions. In this study, we predicted the effects of selected nsSNPs on the PTMs of the CCL21 protein.

Methylation allows PTM to occur in certain proteins, such as when lysine residues are methylated, it impacts the binding of the protein to DNA, which also influences gene expression. GPS-MSP 3.0 was used to determine CCL21 sites that shouldn’t be methylated.

Phosphorylation sites in the CCL21 protein were identified through the GPS 3.0 and NetPhos 3.1. In the GPS 3.0 21 residues (Thr: 26%, serine 63%, and Tyr: 11%) could be phosphorylated. While in Netphose 3.1 16 residues (Thr: 04, Ser: 10, Tyr: 02) could be phosphorylated. For comparison and selection of the normal residue based on GPS 3.0 and NetPhos 3.1 (Table 6).

The prediction of CCL21 ubiquitylation was performed by UbPred and BDM-PUB. CCL21 protein was predicted to have 15 possible sites for ubiquitylation, as detected by BDM-PUB, but UbPred identified just a single potential site of ubiquitylation. The results of ubiquitylation prediction in CCL21 protein are listed in the Table 7.

| Table 6 Phosphorylation prediction CCL21 protein |
| Position | Peptide         | GPS3.0 | NetPhos3.1 |
|          |                 | Kinase  | score  | cutoff | kinase | score |
| 24       | GIPRTQGSDGGAQDC | CK1/VRK2 | 49.038 | 48.397 | DNAPK  | .498  |
| 128      | GCKRTERSQTPKGP* | AGC/GRK/BARK | 186.523 | 65.626 | PKC    | 0.723 |
Table. 7 Ubiquitylation sites Prediction of CCL21 protein

| Peptides         | Position | Score of BDM-PUB (Threshold 0.3) | Score from UbPred (Threshold 0.62) |
|------------------|----------|---------------------------------|----------------------------------|
| GAQDCCLKYSQRKIP  | 34       | 0.88                            | Not Ubiquitylated                 |
| CLKYSQRKIPAKVVR  | 39       | 0.36                            | Not Ubiquitylated                 |
| SQRKIPAKVVRSYRK  | 43       | 2.48                            | Not Ubiquitylated                 |
| KVVRSYRKQEPGLGC  | 50       | 0.90                            | Not Ubiquitylated                 |
| AILFLPRKRSQAEKC  | 68       | 2.47                            | Not Ubiquitylated                 |
| QLMQLHDKTPSPQKP  | 92       | 0.51                            | 0.77                             |
| DKTPSPKPDAQGCRK  | 98       | 1.09                            | Not Ubiquitylated                 |
| KPAQGCRKDRGASKT  | 105      | 2.84                            | Not Ubiquitylated                 |
| RKDRGASKTGKKKGK  | 111      | 3.41                            | Not Ubiquitylated                 |

3.5 Gene-gene Interaction and regulatory SNPs in CCL21

The in-silico tool GeneMANIA depicted that the physical interactions of CCL21 are with CCR7, ACKR4, C114, IGFBP7, CXCL9 and CXCL13 and it also showed that the CCL21 protein is co-expressed with CCL14, TNFSF11, MS4A1, CXCL9, CXCR2, CCR7, CCL19, CXCR6 and CXCL9. In addition, it also indicated that CCL21 is co-localized with CCR6, CCL19, CXCL9 and ACKR4. The pathways of this protein has relation with CCR4, CXCR6, CXCR2, CCR10, CCR7, CCR8, CRR9, CRR3 and C5AR. Moreover, CCL21 was found to have no genetic interactions with other genes. In STRING Predictions, CCL19 is the most interactive gene with CCL21. The presence of CCL21, CCL20, CCL6 and CCR7 are associated with disease in many diseases such as Rheumatoid arthritis, (Viatte et. al., 2013) depicting its significance. Th prediction of gene-gene interactions by
STRING and GeneMANIA are presented in Figure 6 and 7, respectively.

SNPs situated in UTR of the gene not only can impact the miRNA binding sites but also can affect the half-life of RNA and translation of mRNA. Six SNPs in the CCL21 UTR region were identified by MicroSNiPER that might affect the binding of miRNA, whereas PolymiRTS predicted another SNP to affect miRNA binding (total 7 SNPs).

**Table.8 Prediction of effected SNPs in CCL21 Protein regulatory region**

| SNP         | miR ID                                                                 | Score change* |
|-------------|------------------------------------------------------------------------|---------------|
| rs191058724 | hsa-miR-331-5p, hsa-miR-4678, hsa-miR-6509-5p → No pattern            | -0.379        |
| Rs1801937   | hsa-miR-6077, hsa-miR-6830-5p, hsa-miR-1910-3p hsa-miR-2467-3p, hsa-miR-3714 hsa-miR-4257 hsa-miR-6511a-5p | -0.162        |
| Only by PolymiRTS | hsa-miR-342-5p, hsa-miR-4631, hsa-miR-4664-5p, hsa-miR-608, hsa-miR-6134, hsa-miR-6737-5p, hsa-miR-6742-5p, hsa-miR-6812-5p, hsa-miR-6819-5p, hsa-miR-92a-2-5p hsa-let-7a-5p hsa-let-7b-5p hsa-let-7c-5p hsa-let-7d-5p hsa-let-7e-5p hsa-let-7f-5p hsa-let-7g-5p hsa-let-7i-5p hsa-miR-4458 hsa-miR-4500 hsa-miR-98-5p | -0.173        |
| rs41305333  | hsa-miR-1293, hsa-miR-342-5p, hsa-miR-4483, hsa-miR-4651, hsa-miR-4664-5p, hsa-miR-608, hsa-miR-6737-5p, hsa-miR-6747-5p, hsa-miR-6812-5p, hsa-miR-6819-5p, hsa-miR-6890-5p → hsa-miR-1243b-3p hsa-miR-6134 hsa-miR-7854-3p | -0.263        |
| rs147542625 | hsa-miR-1293, hsa-miR-342-5p, hsa-miR-4483, hsa-miR-4651, hsa-miR-4664-5p, hsa-miR-608, hsa-miR-6737-5p, hsa-miR-6747-5p, hsa-miR-6812-5p, hsa-miR-6819-5p, hsa-miR-6890-5p, hsa-miR-876-3, hsa-miR-122-5p hsa-miR-140-5p hsa-miR-4334 hsa-miR-504-3p hsa-miR-6514-5p hsa-miR-8082 | -0.263        |
| rs11574916  | hsa-miR-1293, hsa-miR-342-5p, hsa-miR-4483, hsa-miR-4651, hsa-miR-4664-5p, hsa-miR-608, hsa-miR-6737-5p, hsa-miR-6747-5p, hsa-miR-6812-5p, hsa-miR-6819-5p, hsa-miR-6890-5p, hsa-miR-876-3, hsa-miR-122-5p hsa-miR-140-5p hsa-miR-4334 hsa-miR-504-3p hsa-miR-6514-5p hsa-miR-8082 | -0.263        |
| rs140038884 | hsa-miR-342-5p, hsa-miR-4651, hsa-miR-4664-5p, hsa-miR-608, hsa-miR-6737-5p, hsa-miR-6747-5p, hsa-miR-6812-5p, hsa-miR-6819-5p, hsa-miR-6890-5p → No pattern | -0.15          |
Figure 5 Gene-gene interactions of CCL2 by STRING.
4. Discussion

CCL21 SNPs have been associated with autoimmune thyroid disease (AITD) and rheumatoid arthritis (RA) in several studies [39, 40]. Although several nsSNPs are probably neutral and have little functional effects, many of these nsSNPs have been predicted to be deleterious because due to the disruption of functional sites in proteins or effects on protein’s folding [41]. Therefore, the effects of CCL21 gene SNPs and their association with diseases are critical. The present study showed the result using in silico analysis to determine which nsSNPs are deleterious and what impact they have on the CCL21 protein.

In this study, the SNPs of CCL21 gene were analyzed, which might have a critical role in autoimmune diseases, including RA. The CCL21 gene has only 10% has nsSNPs and 5'UTR SNPs, while the 3'UTR SNPs comprise 3% of the total. In this study we did not include any other SNPs that belonging to
other types of SNPs. Thus, very few SNPs affecting the CCL21 protein directly.

All the tools, used to identify the most damaging nsSNPs, agreed on six nsSNPs that were predicted to have deleterious effect. PROVEAN predicted C75R with -11.422 as the highest score, while R46C with -3.858 as the lowest score among the selected nsSNPs. SNP&GO predicted C75S as the most damaging SNPs having the score of 0.997 as the highest. SIFT predicted L7P with the highest score of 0.04 and R46C, I62N, and V83A with the lowest score of 0.01. Polyphen-2 predicted five nsSNPs with score of 1 on the scale of 0 to 1 and included L7P, D30Y, R46C, C75R, and C75S.

The most damaging nsSNPs, which were selected in this study, were cross-checked several tools in Ensemble genome browser 96, such as Mutation Assessors, CADD, REVEL, and MetalR. CADD, REVEL, and MetalR identified all the selected SNPs (6 nsSNPs) as deleterious and damaging. Mutation Assessor predicted five SNPs as harmful. CADD scores for these nsSNPs were as follows; 18 for L7P and R46S, 26 for I62N, 25 for C75R 25, and 24 for D30Y, C75S, and V83A (CADD score of 30 and 20 mean the SNP is among 0.1% and 1% of the most damaging SNPs in human genome, respectively).

In MutPred1.2, several features are predicted including acetylation gain, Methylation losses, altered interfaces, and intrinsic disorder gain. The C75R and C75S SNPs had the highest $P$-values of 0.950 and 0.906 and followed by L7P, D30Y, and I62N with 0.862, 0.839, and 0.894 respectively. The lowest $P$-values (0.219 and 0.564) were for SNPs R46C and V83A, respectively. The findings suggest that these nsSNPs might affect the CCL21 protein.

For predicting the protein stability, I-Mutant was applied which shows the effects of the nsSNPs. The results are displayed in the form of RI values ranged from 0 to 10, representing the minimum to maximum reliabilities, respectively. The predicted four SNPs that cause lower the protein stability are D30Y, I62N, C75R, and V83A. While the remaining SNPs L7P and R46N were identified to have increased the stability of protein (Table 3). Thus, CUPSAT Server (http://cupsat.tu-bs.de/) was used to cross-check these results to ensure the reliability of the predictions. The I-Mutant prediction was in close agreement with UPSAT server predictions.
In ConSurf, the highly conserved amino acids are predicted to be critical to the protein structure or function as they are based upon amino acid positions in the protein. The structural or functional importance of amino acids is predicted based on the amino acid role in protein [30]. The more the amino acid are conserved in the protein, the more they are considered to have many important function, such as interactions. As acknowledged by Miller and Kumar, nsSNPs that are located in conserved regions are the most damaging [42]. The amino acid positions of the 5 selected nsSNPs were focused on, among which, the D30 was functionally active residue, while the amino acids C75 and V83A were highly conserved, exposed, and structurally active residue and the I62 amino acid was highly conserved and buried. These nsSNPs further confirmed the adverse effect on these selected nsSNPs of CCl21 protein.

The wild type and mutated protein structures of CCl21 protein were modeled using I-TASSER, to which FASTA protein sequences were submitted. It used two template sets, 2nbiA and 2co9, which had 85% and 83% similarity. RAMPAGE values were calculated for the protein structures (using RAMPAGE Server) [43] if they were higher than 80%, then the protein structure was deemed reliable. The RAMPAGE value for the wild type CCl21 protein was 84.8% favored and 15.2% allowed residues. The favored and allowed residues for the mutant D30Y structure were 82.9% and 17.1%, respectively. For the I62N, C73R, C73S, and V83A, the favored residues were 82.6%, 83.2%, 81.2%, and 82.4%, respectively, while the allowed residues were 17.4%, 16.8%, 18.8%, and 16.6% respectively (Morris et al, 1992). All the five selected nsSNPs had high root mean square deviations, indicated that these nsSNPs were the most deleterious SNPs in CCl21 gene. When the RMSD value is greater than 2Å, the differences between mutant and wild-type proteins are greater.

Posttranscriptional modifications (PTMs) are the major factors that drive proteins to perform important functions, such as PPIs and cell signaling [44, 45]. Among the PTM sites in CCl21 protein, the occurrence of PTM sites at these nsSNP positions was investigated. It was noteworthy that none of the phosphorylation and ubiquityation sites were located at the most damaging nsSNPs sites. Other nsSNPs positions were also investigated using GPS3.0 and NetPhos3.1, which showed 7 phosphorylation sites at the nsSNPs positions (Table 4). Ubiquitylation sites were predicted by BDM-PUB and
UbPred. BDM-PUB detected 15 sites and UbPred predicted one ubiquitylation site at 92. The STRING and GeneMANIA predictions indicated that CCL19 was the most interactive protein with CCl21. However, RA is associated with many proteins, such as CCL20, CCL21, CCR7 and TRAF3IP3, indicating the importance of CCL21 protein in RA (Viatte, Plant & Raychaudhuri 2013). Thus, it can be inferred that any of the most destructive nsSNPs in the gene CCL21 might ultimately affect and interrupt the normal function of other expressive genes based on their interaction patterns and their co-expression profile with many proteins, such as CCL20, CCL21, CCR7, and TRAF3IP3.

Conclusions

This study suggests that various nsSNPs can disturb the structure and/or function of CCL21 protein. In native protein of CCL21 gene, five major mutations found were Aspartic acid to Tyrosine at position 30 (rs753133670), Isoleucine to Asparagine at position 62 (rs1170851787), Cysteine to Arginine at position 75 (rs759733358), Cysteine to Serine at position 75 (rs776954599) and Valine to Alanine at position 83 (rs1182863895). These results supported by high MutPred 1.2 and high RMSD P values that also indicate that these nsSNPs have a significant role in the onset of RA and other related diseases. Thus, these SNPs can be considered as critical candidates for the development of diseases associated with CCl21 dysfunction, ultimately helping to discover effective drugs and create precision medicine. Laboratory experiments are required to investigate the effect of these polymorphisms on protein structure and function. Furthermore, the use of various animal for studying CCL21 mutation can be very helpful when studying disease development.

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