RESEARCH

In-silico analysis unravels the structural and functional consequences of non-synonymous SNPs in the human *IL-10* gene

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Abstract

Background: Interleukin-10 (IL-10) is an anti-inflammatory cytokine that affects different immune cells. It is also associated with the stimulation of the T and B cells for the production of antibodies. Several genetic polymorphisms in the *IL-10* gene have been reported to cause or aggravate certain diseases like inflammatory bowel disease, rheumatoid arthritis, systemic sclerosis, asthma, etc. However, the disease susceptibility and abnormal function of the mutated IL-10 variants remain obscure.

Results: In this study, we used seven bioinformatics tools (SIFT, PROVEAN, PMut, PANTHER, PolyPhen-2, PHD-SNP, and SNPs&GO) to predict the disease susceptible non-synonymous SNPs (nsSNPs) of *IL-10*. Nine nsSNPs of *IL-10* were predicted to be potentially deleterious: R42G, R45Q, F48L, E72G, M95T, A98D, R125S, Y155C, and I168T. Except two, all of the putative deleterious mutations are found in the highly conserved region of IL-10 protein structure, thus affecting the protein's stability. The 3-D structure of mutant proteins was modeled by project HOPE, and the protein–protein interactions were assessed with STRING. The predicted nsSNPs: R42Q, R45Q, F48L, E72G, and I168T are situated in the binding site region of the IL-10R1 receptor. Disruption of binding affinity with its receptor leads to deregulation of the JAK-STAT pathway and results in enhanced inflammation that imbalance in cellular signaling. Finally, Kaplan–Meier Plotter analysis displayed that deregulation of *IL-10* expression affects gastric and ovarian cancer patients' survival rate. Thus, *IL-10* could be useful as a potential prognostic marker gene for some cancers.

Conclusion: This study has determined the deleterious nsSNPs of *IL-10* that might contribute to the malfunction of IL-10 protein and ultimately lead to the IL-10 associated diseases.

Keywords: Interleukin-10, JAK-STAT pathway, nsSNPs, Prognostic marker, Polymorphisms

Background

The immune system is constituted of various immune cells, which are responsible for monitoring and getting rid of unfamiliar agents or invading microorganisms. The immune cells can act directly by themselves or by synthesizing molecules capable of inducing B cells, NK cells, T cells, and other immune cells [1, 2]. Activation and differentiation of the immune cells are largely dependent on different types of interleukins (ILs). Interleukins are the subsets of a large group of naturally occurring cytokines that are primarily released from specific immune cells in response to endotoxic threat, stress, heat, or inflammation. They act as cellular messengers by binding to high-affinity receptors on the cell surface [3, 4]. ILs play essential roles in innate and adaptive immune systems and modulate cell behavior [4, 5]. Interleukin 10 (IL-10) is one of the most crucial anti-inflammatory cytokines

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with the most diverse immune cells' effects [6]. It is produced by activated immune cells, particularly monocytes /macrophages and T cell subsets, including Tr1, Treg, and Th1 cells. IL-10 can down-regulate the expression of pathogenic Th17 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages [4, 7]. In addition to the immune suppression role, the IL-10 can also play an immune stimulatory role for B and T cells [6]. This dimeric cytokine's pleiotropic activities are mediated by its interaction with the tetrameric cell surface receptor complex, consisting of two IL-10R1 and two IL-10R2 [8, 9]. The receptor complex assembles sequentially: first, IL-10 binds with high-affinity IL-10R1 cell surface receptor and form IL-10/IL-10R1 complex [10, 11]. This IL-10/ IL-10R1 intermediate complex is subsequently recognized through the low-affinity IL-10R2 receptor, resulting in an active signaling complex that induces the intracellular JAK-STAT pathway [12]. Recent studies showed that immune-related genes like *IL-10* are highly polymorphic and associated with various types of diseases [13–15]. The most frequently occurred polymorphism is single nucleotide polymorphism (SNP) and can be identified once in every 100–300 base pairs of the human genome. It has been estimated that nearly 10 million SNPs are present in the human genome where 0.5 million SNPs are located in the coding region of different genes [16–19]. A recent report on Trans Omics for Precision Medicine (TOPMed) program suggested an average of 3.78 million genetic variants are present in each genome. Among the all genetic variants, a total of 23,916 variants or SNPs are coding variation [20]. The SNPs that may alter amino acid residue in the protein sequence are known as Non-synonymous SNPs (nsSNPs). They are particularly important as they may affect the protein function by destabilizing protein structure or altering its physicochemical properties [21]. Non-coding SNPs are also important as they may influence mRNA splicing, binding of the transcription factor to *cis*-regulatory elements, differential expression of genes, degradation of mRNA, and alternation in the sequences of noncoding RNAs [22]. In the recent era, hundreds and thousands of SNPs were associated with hundreds of disease studies [23, 24]. In various studies, several polymorphisms are identified in the coding and noncoding regions of *IL-10*. Some of the SNPs of *IL-10* have already been characterized and found to significantly influence the immune response toward pathogenic challenges and disease outcome [25]. These polymorphisms cause functional changes of the *IL-10* protein that are associated with various inflammatory and autoimmune diseases, such as inflammatory bowel disease: Crohn's disease and ulcerative colitis, chronic hepatitis B and C, allergy and autoimmunity [25–27]. Blockage of *IL-10* signaling may lead to enhanced inflammation

and an increased number of Tregs (Regulatory T-cells) and MDSCs (Myeloid Derived Suppressor Cells), which inhibit tumor immunity, allowing tumors to grow [28]. Individuals with Chronic inflammatory bowel disease are predisposed to colon cancer, and individuals with chronic hepatitis are more prone to develop hepatocellular carcinoma [29, 30]. Considering the importance of *IL-10* in multiple diseases, our present study investigates the disease-causing nsSNPs in the *IL-10* gene and determining their deleterious effects on the protein. High-risk deleterious SNPs were further analyzed computationally to predict their structural and functional impact on IL-10 protein, which provides new insights for further genetic association studies.
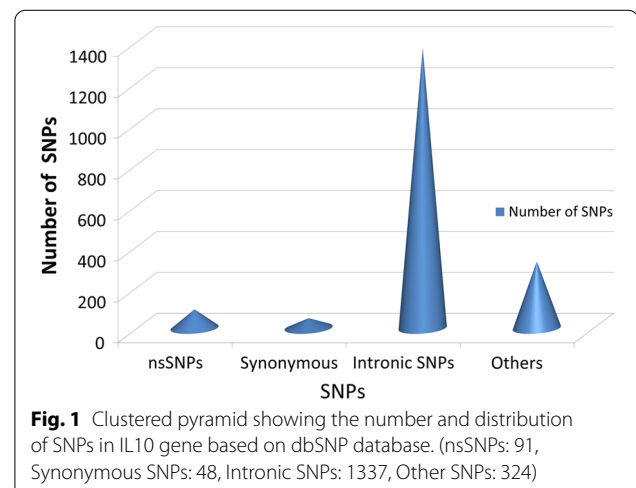
Materials and methods
Retrieval of *IL-10* nsSNPs (dataset)
The entire reported SNPs of the *IL-10* gene and its protein sequence (Uniprot ID P22301) were retrieved from NCBI dbSNP (http://www.ncbi.nlm.nih.gov/snp) and Uniprot Knowledgebase database (http://www.uniprot.org/), respectively. A total of 1800 SNPs of different functional classes (Fig. 1) were mapped in *IL-10* gene sequence. Out of 1800 SNPs, 91 are non-synonymous SNPs (nsSNPs) found in the coding region that may lead to missense or nonsense mutations, subsequently affecting the protein's structure and function. Our investigation accounted for the nsSNPs in the coding region of IL-10 Protein.

Prediction of the deleterious nsSNPs
We employed seven different tools to predict the deleterious effects of nsSNPs: SIFT-Sorting Intolerant From Tolerant (SIFT; http://sift.bii.a-star.edu.sg/) [31], Protein Variation Effect Analyzer (PROVEAN; http://provean.

Fig. 1 Clustered pyramid showing the number and distribution of SNPs in IL10 gene based on dbSNP database. (nsSNPs: 91, Synonymous SNPs: 48, Intronic SNPs: 1337, Other SNPs: 324)

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jcvi.org/index.php) [32] Predictor of human Deleterious Single Nucleotide Polymorphisms (PhD-SNP; http://snps.biofold.org/phd-snp/phd-snp.html) [33] PMut (http://mmb.irbbarcelona.org/PMut), Polymorphism Phenotyping v2 (PolyPhen-2; http://genetics.bwh.harvard.edu/pph/) [34], Protein analysis through evolutionary relationship (PANTHER; http://www.pantherdb.org/tools/csnpScoreForm.jsp) [35] and SNPs&GO (http://snps.biofold.org/snps-and-go/snps-and-go.html) [31–36]. SIFT predicts an amino acid substitution's effects on the function of a protein based on sequence homology and the substituted amino acids' physical characteristics [37]. PolyPhen-2 tool predicts the effects of substituted amino acids on structure and function of a protein based on physical and comparative properties [34]. PROVEAN is a support vector machine-based (SVM) server, which predicts whether a substituted amino acid has an impact on the function of a given protein or not. The tools like PANTHER, SNPs&GO, PHD- SNP, and PMut were used for the prediction of whether a single nucleotide polymorphism is likely to be involved in the insurgence of diseases using functional annotation of protein [35, 38–40]. The nsSNPs predicted deleterious by at least six of the above mentioned in silico tools were considered as high risk nsSNPs and selected for the further analysis.

Analyzing protein stability due to mutations

I-Mutant 3.0 (http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi), Mupro (http://mupro.proteomics.ics.uci.edu) and INPS-MD (http://inpsmd.biocomp.unibo.it) tools were used to evaluate the stability changes of IL-10 protein upon point mutations. I-Mutant 3.0 is a support vector machine (SVM) based predictor that determines the degree of protein destabilization and measures the $\Delta\Delta G$ value (kcal/mol). The $\Delta\Delta G$ (delta delta G) value is the difference between Gibbs free energy values of mutated protein from the Gibbs free energy value of wild type protein. A $\Delta\Delta G$ value less than '0' indicates that the variants cause the decreased stability of the protein whether $\Delta\Delta G$ value greater than '0' means increased stability of that protein [41].

On the other hand, MUpro uses a large number of mutation datasets which actually based on both SVM and neural networks machine learning methods. The other third tools INPS-MD (Impact of Non-synonymous mutations on Protein Stability-Multi Dimension) based on sequence descriptor that uses Support Vector Regression (SVR) to calculate $\Delta\Delta G$ value. Both the MUpro and INPS-MD measure the $\Delta\Delta G$ for estimation of protein stability and the cut-off value of $\Delta\Delta G$ is also same to I-Mutant 3.0 [42, 43].

The IL-10 protein sequence along with wild type and substitute amino acids at their corresponding position was used as input in the aforementioned tools to predict the mutational effect on protein stability [41].

Identification of mutational impacts on structural and functional properties of proteins

For sorting out disease associated or neutral amino acid substitutions in protein sequence, the commonly predicted mutations were further examined by MutPred2 web server (http://mutpred.mutdb.org). It is a machine learning based tool that integrates genetic and molecular data for prediction of the pathogenicity of substituted amino acid. It also predicts molecular cause of the disease [44].

Conservation profile of high-risk nsSNPs

nsSNPs, which are positioned at highly conserved regions tend to be more deleterious than the nsSNPs those are situated at non-conserved sites. By using empirical Bayesian inference, ConSurf web server predicts putative structural and functional amino acid residues and estimates evolutionary conservation based on the phylogenetic relations between homologous sequences [45]. To further investigate the potential effects of the high-risk nsSNPs, we calculated the degree of evolutionary conservation at all amino acid sites in the IL-10 protein using the ConSurf web server.

Predicting the molecular effects of high risk nsSNPs on protein structure

Project HOPE is an automatic mutant analysis server that helps to analyze the structural and biochemical effects of a point mutation in a protein sequence [46]. We submitted the nine SNPs (rs ID) with the primary structure of IL-10 protein from Protein Data Bank (http://www.rcsb.org/pdb/) into HOPE. HOPE predicts the 3D structure of the mutated protein by collecting structural information from a series of sources and gives the explanation of such a change (in both structure and function of the protein).

Protein–protein interaction prediction

Protein–protein interactions are studied to unveil and annotate all functional interactions among cell proteins. The online database STRING (STRING; http://string-db.org/) was used to predict protein- protein interactions [47].

Kaplan–Meier plotter analysis

Kaplan–Meier plotter database (http://kmplot.com/analysis) uses Gene Expression Omnibus (GEO), European Genome Phenome Atlas (EGA), and the Cancer Genome Atlas (TCGA) datasets for the relapse free and overall survival (OS) information that offers meta-analysis-based discovery and biomarker assessment in cancer patients.

The aim of this analysis is to estimate the time to death, an event will eventually occur in everyone that may have significant implications when using these estimates to inform clinical decisions, health care policies, and resource allocation [48]**.** In this algorithm, the potential effects of 54,675 genes (mRNA, miRNA, protein) can be examined on survival of 13,316 cancer patients (comprising 6234 breast, 3452 lungs, 1,440 gastric, and 2190 ovarian cancer) through microarray gene expression data of 21 types of cancers [49]. By using 207,433 at Affymetrix ID of *IL-10* gene, the overall survival analysis was performed on total 13,316 cancers patients. The hazard ratio (HR) with 95% confidence intervals and log rank P-value were enumerated and displayed on the plot.

Results

nsSNPs retrieved from dbSNP database

In the dbSNP database showed the human *IL-10* gene consists a total of 1800 SNPs, of which 91 were nsSNPs/missense leading to amino acid substitution (5%), 1336 were intronic SNPs (74%), 48 were synonymous SNPs (3%), and the rests were of other types (Fig. 1). We selected only the nsSNPs for our investigation (Additional file 1: Table S1).
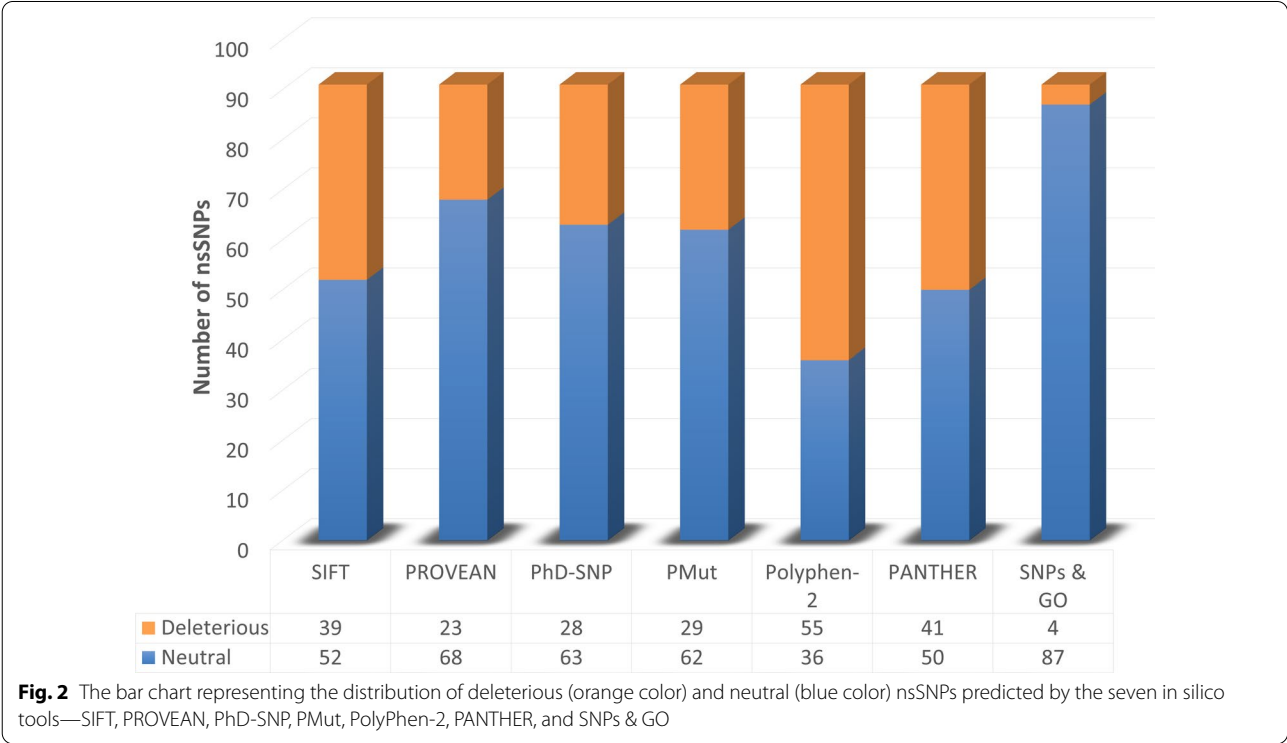
Prediction and analysis of deleterious nsSNPs

The functional impact of nsSNPs was assessed by evaluating the importance of amino acids they alter. A dataset of a total of 91 polymorphic inputs was used for analysis. Structural and functional effects of deleterious SNPs on the IL-10 protein were screened by various computational tools. A graphical representation of the deleterious nsSNPs predicted by seven different computational tools is illustrated in Fig. 2.

First, 91 nsSNPs of *IL-10* were submitted to SIFT algorithm. According to SIFT result, out of 91 nsSNPs, 39 nsSNPs were predicted intolerant whose TI scoring was ≤ 0.05. 11 SNPs showed a highly deleterious effect with a tolerant index (TI) score of 0.00; 13 SNPs showed 0.01 TI score, and the remaining 15 SNPs TI score was 0.02–0.04 (Additional file 1: Table S2).

Among the other tools, PROVEAN predicted 23 nsSNPs (out of 91 nsSNPs) as "Deleterious", similarly, PhD-SNP and PMut tools proposed 28 and 29 nsSNPs, respectively, as "Disease" (Additional file 1: Tables S3, S4, and S5). Additionally, PolyPhen-2 predicted 13 nsSNPs as "Possibly damaging" and 42 nsSNPs as "Probably damaging" (Additional file 1: Table S6). Moreover, PANTHER_PSEP foretold 41 SNPs as "Deleterious". Among them, 28 SNPs were identified as possibly damaging, and the remaining 13 SNPs were identified as "Probably damaging" (Additional file 1: Table S7)**.** In SNPs&GO tools, five nsSNPs were predicted to be associated with various types of diseases, and the rest of the 86 SNPs were not predicted to have any effects (Additional file 1: Table S8)**.** Finally, mutations predicted deleterious/ damaging /

Fig. 2 The bar chart representing the distribution of deleterious (orange color) and neutral (blue color) nsSNPs predicted by the seven in silico tools—SIFT, PROVEAN, PhD-SNP, PMut, PolyPhen-2, PANTHER, and SNPs & GO

	SIFT	PROVEAN	PhD-SNP	PMut	Polyphen-2	PANTHER	SNPs & GO
Deleterious	39	23	28	29	55	41	4
Neutral	52	68	63	62	36	50	87

disease-related effects by at least six of the analyzed in silico tools were considered for further investigation (Table 1).

Computational analysis by the seven mentioned tools exhibited nine highly damaging nsSNPs in the *IL-10* gene. Out of nine nsSNPs, three of them (e.g., rs550164520 R45Q, rs1421978042 A98D, and rs1022828778 Y155C) were predicted deleterious unanimously by all the employed tools, and other six nsSNPs (rs1274280163 R42G, rs745923816 F48L, rs545228684 E72G, rs1354773439 M95T, and rs1310781150 R125S) were predicted deleterious by the at least six computational tools. Except for the R42G, the remaining eight of them are novel SNPs.

Identification of functional and structural modifications of IL-10 predicted by MutPred2

The shortlisted nine nsSNPs predicted as deleterious from the previous steps were submitted to the MutPred2 web server. The resulting probability scores, g-value, and p-value are shown in Table 2. It helps to predict the reason for molecular alternations potentially affecting the phenotype. The structural and functional alterations predicted include- loss of sulfation, acetylation, allosteric site; alerted transmembrane protein, coiled-coil, disordered interface, metal-binding; and gain of solvent accessibility, intrinsic disorder, loop, B-factor, catalytic site. The output of MutPred2 tool consists of a general score (g) that represents the average score from all neural networks in MutPred2. The threshold value of 'g' score is 0.50. A 'g-score' value greater than 0.50 (g > 0.50) for a certain mutation suggest the pathogenicity. [44]. Scores with g-value > 0.5 and p-value < 0.05 are referred to as actionable hypotheses, whereas the scores with g-value > 0.75 and p-value < 0.05 are referred to as confident hypotheses. In MutPred2 prediction, the F48L, M95T, A98D, R125S, Y155C, and I168T substitution

showed g-values greater than 0.5 and p-values lower than 0.05 (Table 2).These predicted data provide compelling evidence that the several nsSNPs could play a potential role in the structural and functional modifications of IL-10 protein.

The impact of predicted deleterious mutations on IL-10 protein stability

The nine predicted nsSNPs were further subjected to I-Mutant 3.0, INPS- MD and Mupro tools for protein stability analysis through comparing free energies. Seven of the nine nsSNPs (R42G, R45Q, F48L, E72G, M95T, A98D, and I68T) showed a decrease in structure stability unanimously with all the three analyzed tools. The four variants R42G, R45Q, F48L, and I168T unanimously showed ΔΔG (delta delta G) values less than -1 kcal/mol. The others three variants E72G, M95T, and A98D unanimously showed the ΔΔG values less than zero, which would be predicted to alter the structure and function of the protein by decreasing its stability (Table 3).

Analysis of conservation

Evolutionary conserved amino acids of a protein across species are functionally and biologically very important. Mutation in the conserved region of a protein are often deleterious and may affect the function of the protein. Our ConSurf analysis showed that there are a total of 72 conserved amino acid in the IL-10 protein with scores between 7 and 9. Out of the nine selected high-risk SNPs in IL-10 protein, seven nsSNPs (R45Q, F48L, M95T, A98D, R125S, Y155C, and I168T) were located in the highly conserved region with conservation value ranging 7–9 (Fig. 3). The other two nsSNPs (R42G and E72G) were situated in the variable region. Taken together, our data strongly suggest that the selected nsSNPs are deleterious to IL-10 structure and/or function.

Table 1 High risk nsSNPs predicted by minimum six or more in silico programs

SNP ID	Amino acid change	SIFT	PANTHER	PROVEAN	PhD-SNP	PMut	PolyPhen-2	SNPs&GO
rs1274280163	R42G	D	D	D	D	D	D	N
rs550164520	R45Q	D	D	D	D	D	D	D
rs745923816	F48L	D	D	D	D	D	D	N
rs545228684	E72G	D	D	D	D	D	D	N
rs1354773439	M95T	D	D	D	D	D	D	N
rs1421978042	A98D	D	D	D	D	D	D	D
rs771912629	R125S	D	D	D	D	D	D	N
rs1022828778	Y155C	D	D	D	D	D	D	D
rs1310781150	I168T	D	N	D	D	D	D	D

*Here, "D" indicates disease and "N" indicates neutral

Table 2 Functional and structural modifications of IL-10 predicted by MutPred2. Here 'g-value' stands for general score of MutPred2

| SNPs	Actionable/confident hypothesis	g-value	*p* value	Probability
F48L	Gain of relative solvent accessibility	0.705	0.01	0.29
	Altered transmembrane protein		0.0082	0.26
	Altered coiled coil		0.03	0.13
L91V	Gain of loop	0.506	0.02	0.27
	Altered transmembrane protein		0.0025	0.23
	Gain of sulfation at Y 90		0.04	0.02
M95T	Altered transmembrane protein	0.732	0.00085	0.27
	Loss of sulfation at Y 90		0.04	0.01
A98D	Gain of intrinsic disorder	0.612	0.04	0.31
	Gain of loop		0.0086	0.29
	Gain of B-factor		0.02	0.26
	Altered transmembrane protein		0.0051	0.20
R125S	Altered disorder interface	0.543	0.01	0.30
Y155C	Altered metal binding	0.764	0.00046	0.50
	Altered Ordered interface		0.0044	0.36
	Altered Disordered interface		0.0074	0.36
	Altered Transmembrane protein		0.000063	0.32
	Loss of Relative solvent accessibility		0.0059	0.32
	Loss of Acetylation at K152		0.0078	0.27
	Loss of Allosteric site at E160		0.03	0.23
	Gain of Catalytic site at E160		0.04	0.11
I168T	Altered Disordered interface	0.512	0.0026	0.44
	Altered Ordered interface		0.02	0.30
	Gain of Relative solvent accessibility		0.02	0.27
	Altered Transmembrane protein		0.04	0.10
	Altered Coiled coil		0.04	0.10
	Gain of N-linked glycosylation at N166		0.04	0.02

Table 3 Protein stability change prediction using I-Mutant 3.0, MUpro, and INPS-MD. AA = amino acid

AA mutation	I-Mutant 3.0		MUpro		IPNS-MD	
	Stability	ΔΔG (kcal/mol)	Stability	ΔΔG (kcal/mol)	Stability	ΔΔG (kcal/mol)
R42G	Decrease	− 1.68	Decrease	− 1.269029	Decrease	− 1.40964
[R45Q	Decrease	− 1.28	Decrease	− 1.1934308	Decrease	− 1.21672
F48L	Decrease	− 1.49	Decrease	− 1.3723104	Decrease	− 1.95635
E72G	Decrease	− 0.82	Decrease	− 1.9791607	Decrease	− 1.06295
M95T	Decrease	− 0.81	Decrease	− 1.1092068	Decrease	− 1.81955
A98D	Decrease	− 0.95	Decrease	− 0.74453775	Decrease	− 1.38504
R125S	Decrease	− 0.96	Decrease	− 1.0951197	No result	No result
Y155C	Increase	− 0.93	Decrease	− 0.64707048	Decrease	1.59442
I168T	Decrease	− 2.14	Decrease	− 1.8132602	Decrease	− 3.01639

Protein structure analysis

The 3D model structures of the nine mutated IL-10 proteins were generated by Project HOPE (http://www.cmbi.ru.nl/hope/) (Fig. 4). Project HOPE simulates the structural characteristics of amino acid residues substitutions on native protein. Besides, project HOPE showed the physicochemical properties such as size, charge, hydrophobicity values differed between wild and mutant type amino acids, as shown in Table 4. All the nine predicted nsSNPs caused changes in the size of amino acids. Apart

Fig. 3 Analysis of evolutionary conservancy of IL-10 by Consurf (UniProt ID P22301)
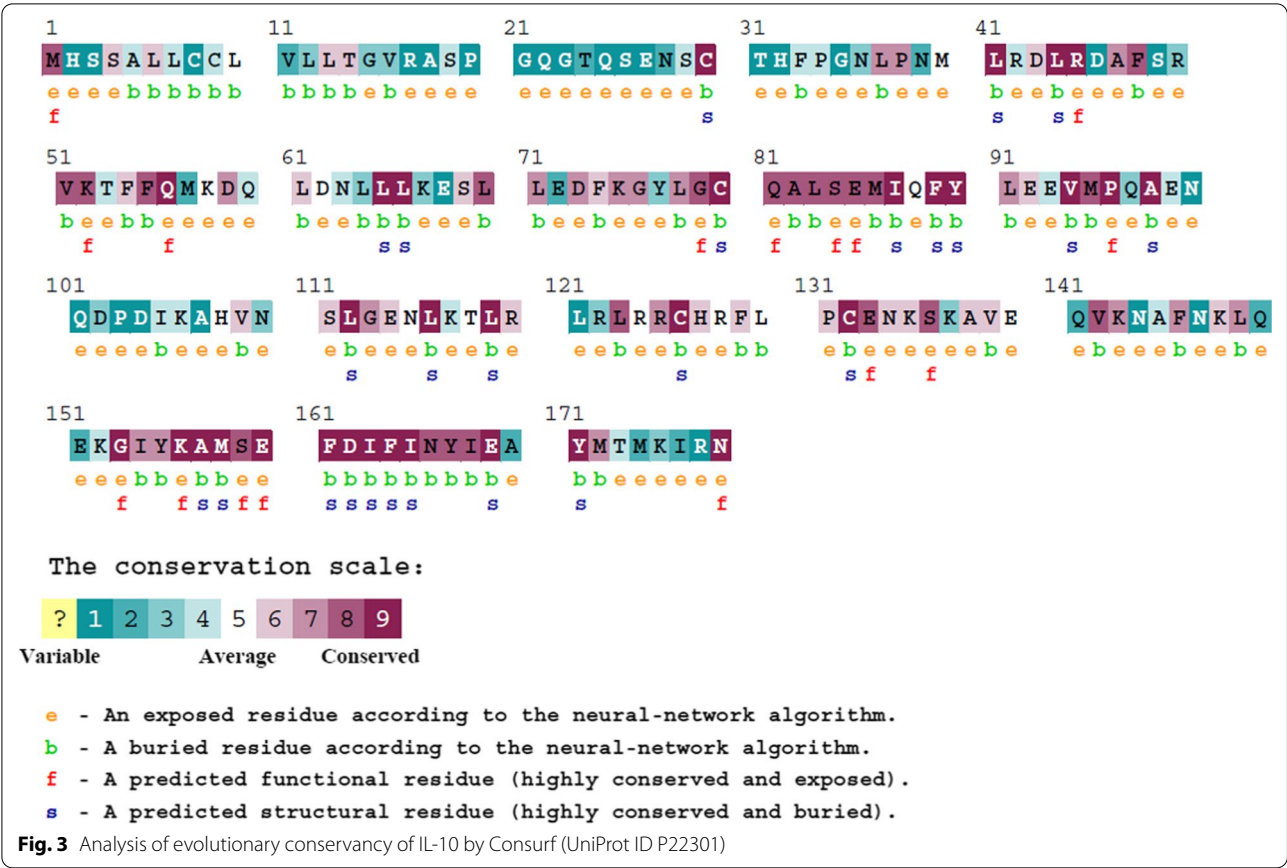
from the A98D mutation, the size of the remaining eight mutant amino acids become smaller than the wild type variant. Out of nine nsSNPs, five nsSNPs (R42G, R45Q, E72G, A98D, and R125S) found to alter the amino acid charges in the mutant variant. Also, seven of the nsS-NPs (R42G, E72G, M95T, A98D, R125S, Y155C, and I168T) caused changes in hydrophobicity of amino acids (Table 4).

Further analysis with HOPE project found that out of the nine mutations, four (R42G, R45Q, F48L, and R125S) were situated in the domain region. Besides, four mutations (R42G, E72G, R125S, and Y155C) were found to cause the loss of hydrogen bond interaction and three (M95T, A98D, and I168T) caused the loss of hydrophobic interaction. Interestingly, all of the nine mutations were found to locate in the conserved region that might affect the structure and function of IL-10 protein (Table 5).

Protein–protein interaction analysis

The STRING server result showed that Interleukin-10 protein interacts with ten proteins including, interleukin-10 receptor alpha subunit (IL-10RA), interleukin-6 (IL6), tumor necrosis factor (TNF), interleukin-1 beta (IL1B), Interleukin-8 (CXCL8), C–C motif chemokine-2 (CCL2), signal transducer and activator of transcription-3 (STAT 3), Granulocyte–macrophage colony stimulating factor (CSF2), C–C motif chemokine-5 (CCL5), and T-lymphocyte activation antigen CD80 (CD80) (Fig. 5).

Clinical correlation between IL-10 deregulation and the survival rate of patients with different cancer types

In this step, we attempted to associate the deregulation of the *IL-10* gene with clinical databases to infer possible functional consequences of *IL-10* deregulation in cancer patients. Kaplan–Meier Plotter was used to retrieve the prognostic information of *IL-10* gene and analyzed with the survival of patients with gastric, lungs, breast, and ovarian cancer that is shown in Fig. 6. The plot analysis revealed that *IL-10* deregulation shows different implications in different cancer types. In the case of gastric and ovarian cancer, the increased level of *IL-10* expression predicted decreased number of patients at risk (more survival rate). The HR ratio and P-value for gastric and ovarian cancer were (HR 1.37 [1.14–1.64], P = 0.00078) and (HR 1.22 [1.08–1.39], P = 0.0017) (Fig. 6). In addition, for breast (HR 0.85 [0.75–0.97], P = 0.013) and lung (HR 1.84 [1.52–2.24], P = 0.00000000028) cancer, a

Modeled ribbon structure of mutated IL-10	Close-up view of the mutated site of IL-10
rs1274280163 R42G	
rs550164520 R45Q	
rs745923816 F48L	
rs545228684 E72G	
rs1354773439 M95T	
rs1421978042 A98D	
rs771912629 R125S	
rs1022828778 Y155C	
rs1310781150 I168T	

Fig. 4 Prediction of 3-D model structure of IL-10 protein by using Project HOPE. Here, violet color on the ribbon diagram represents the site of mutation. Green and red colors indicate native and mutated amino acids, respectively

low level of *IL-10* expressions is associated with a high number of patients at risk (Less survival rate). A control expression of the *IL-10* gene appears to be inevitable for a healthy person. An erroneous transcription of the *IL-10* gene could lead to the development of different types of cancer. Therefore, the *IL-10* gene could be advantageous as a putative prognostic marker for some cancers. Since

the nsSNPs have an impact on IL-10 protein's structure and function, we believe that the nine nsSNPs identified in this study are expected to have almost similar functional consequences in *IL-10* deregulation.

Discussion

The human *IL-10* gene is located on Chromosome-1 that encodes 178 amino acid long protein. After cleavage of the N-terminal 18 amino acid signal sequence, the mature protein consists of 160 amino acids [8, 50, 51]. Thousands of polymorphisms have already been reported in the both coding and noncoding region of the *IL-10* gene. Identification of functionally important SNPs from a pool containing both damaging and neutral SNPs with molecular approaches seems to be expensive and time-consuming. Multiple computational approaches play a great role in predicting and identifying important variants that have damaging effects on proteins structure and function [52–54]. However, the present in silico approaches have some weaknesses in prediction of deleterious nsSNPs because every algorithms use different parameters for prediction. Thereby, single algorithms should not be considered for proper prediction of deleterious nsSNPs. In order to predict deleterious nsSNPs precisely requires implementation of different algorithms with different parameters and aspects. A consensus result obtained from the majority of the tools can provide a reliable outcome. In this study, we investigated the genetic variations in *IL-10* locus. Nine high-risk missense SNPs were identified by seven different computational tools amid 91 missense SNPs that have been reported to date. The filtered nine nsSNPs were analyzed in I-Mutant 3.0, Mupro and IPNS-MD to investigate their protein stability effects. Seven of the nsSNPs were found to cause a decrease in stability, whereas the two others predicted to increase the rigidity of IL-10 protein (Table 3). We have predicted the conserved amino acid residues in the IL10 protein based on evolutionary conservation using ConSurf. The ConSurf results revealed that most of the high risk nsSNPs position located in a highly conserved region (Fig. 3). The reason of molecular alternation that potentially affects the structure and function of the IL10 protein were examined using MutPred2 web server (Table 2). Alternation of protein's stability affects the conformational structure and thus governs the function of a protein [55]. The aforementioned nsSNPs affect the proteins stability and might cause maximum damaging effects on its structure and function. Decreased protein stability may change the protein folding mechanism and can cause increased degradation or aberrant aggregation of proteins [56, 57]. Project HOPE software results have provided important information about the possible effects of missense SNPs of *IL-10*

Table 4 Results of wild-type and mutant-type amino acid properties obtained from Project Hope software

Amino Acid change	Wild type amino acids			Mutant type amino acid		
	Size	Charge	Hydrophobicity	Size	Charge	Hydrophobicity
R42G	Larger	Positive	Less	More smaller	Neutral	More hydrophobic
R45Q	Larger	Positive	–	More smaller	Neutral	–
F48L	Larger	–	–	More smaller	–	–
E72G	Larger	Negative	Less hydrophobic	More smaller	Neutral	More Hydrophobic
M95T	Larger	–	More hydrophobic	More smaller	–	Less hydrophobic
A98D	Smaller	Neutral	More hydrophobic	More larger	Negative	Less hydrophobic
R125S	Larger	Positive	Less hydrophobic	Smaller	Neutral	More hydrophobic
Y155C	Larger	–	More hydrophobic	Smaller	–	More hydrophobic
I168T	Larger	–	More hydrophobic	Smaller	–	Less Hydrophobic

gene. The polymorphisms (rs1274280163, rs550164520, rs745923816, rs545228684, rs1354773439, rs1421978042, rs771912629, rs1022828778, and rs1310781150) result in R42G, R45Q, F48L, E72G, M95T, A98D, R125S, Y155C, and I168T amino acid substitutions, respectively. Those substituted amino acids have different physiochemical properties that may interrupt the IL-10 protein structure. Due to the polymorphisms, the mutated residues (R42G, E72G, R125S, and Y155C) were more hydrophobic than wild-type residues, which might cause the loss of Hydrogen bond with other molecules and may disrupt correct protein folding. In contrast, the wild-type amino acid residues were more hydrophobic than in A98D and I168T mutation, resulting in loss of hydrophobic interactions with other molecules on the surface of the protein.

It is well established that the human IL-10 protein is a tight dimer consisting of two interpenetrating subunits. Each of the subunits of IL-10 protein comprises six alpha-helices named A-F. The dimeric structure of IL-10 is mainly stabilized by the intertwining of helices E (amino acid position 118 to 131) and F (amino acid position 133 to 159) across the subunit interface [50]. Any mutation within the E and F helix regions of IL-10 subunits could affect the dimerization process of IL-10 protein. Interestingly, R125S and Y155C polymorphisms are found to be located in the E and F helices of IL-10 subunits, respectively. From our HOPE project analysis, we observed that R125S and Y155C mutations cause loss of hydrophobic interaction. Additionally, R125S mutation is found to be responsible for amino acid charge alternation. This finding indicates R125S and Y155C mutations could interfere with the intertwining of two subunits and thereby with the dimerization process of IL-10. Furthermore, the polymorphisms R42G, R45Q, F48L, R125S, and Y155C are located in the protein catalytic domain and are crucial for its catalytic function. Mutation of these residues might disrupt the catalytic activity of IL-10. In the wild

type IL-10 protein, the amino acids M95 and A98 residues produce an alpha-helix structure (annotated from UniProt). However, the M95T and A98D polymorphisms of IL-10 do not support the alpha-helix as a secondary structure in the respective position. The other two mutations R42G and E72G introduced a glycine residue at these positions. Glycine is very flexible and can disrupt the required rigidity of the protein at this position. The overall results showed that the modeled mutated protein (Fig. 4) is different from wild-type IL-10 protein, resulting in destabilization of the protein and can cause defective binding of IL-10 with its receptor. The mature IL-10 protein harbors three regions, namely region A, B, and C. The region A (LRDLRDAFSRV is in the position no 23 to 33 amino acids), region B (FFQMKDQLDNLLLKES-LLE is in 36 to 54 position), and region C (DIFINY-IEAYMTMKIRN amino acid positions 144 to 160) are binding site regions that interact with the IL-10R1 receptor [58]. Our findings showed that out of nine high-risk nsSNPs, five are (R42G, R45Q, F48L, E72G, and I168T) mainly situated in the binding site regions. A previous study by Yoon et al. reported previously that the R42G mutation of IL-10 causes an 80% loss of binding affinity with IL-10R1 [59]. Therefore, we can speculate that the mutations in the binding site regions of IL-10 may disrupt their interactions with their respective receptors, ultimately preventing the downstream signaling by IL-10. Blockage of IL-10 signaling may lead to enhanced inflammation and increased number of Tregs and MDSCs, which inhibit tumor immunity, allowing tumors to grow [28]. Long-term enhanced inflammation contributes to tumor initiation and progression [29]. The other predicted four nsSNPs (R45Q, F48L, E72G, and M95T) would be responsible for the loss of protein function, which may result in the deregulation of the JAK-STAT pathway, causing an imbalance in cellular signaling. The mutations caused by those high risk nine nsSNPs may
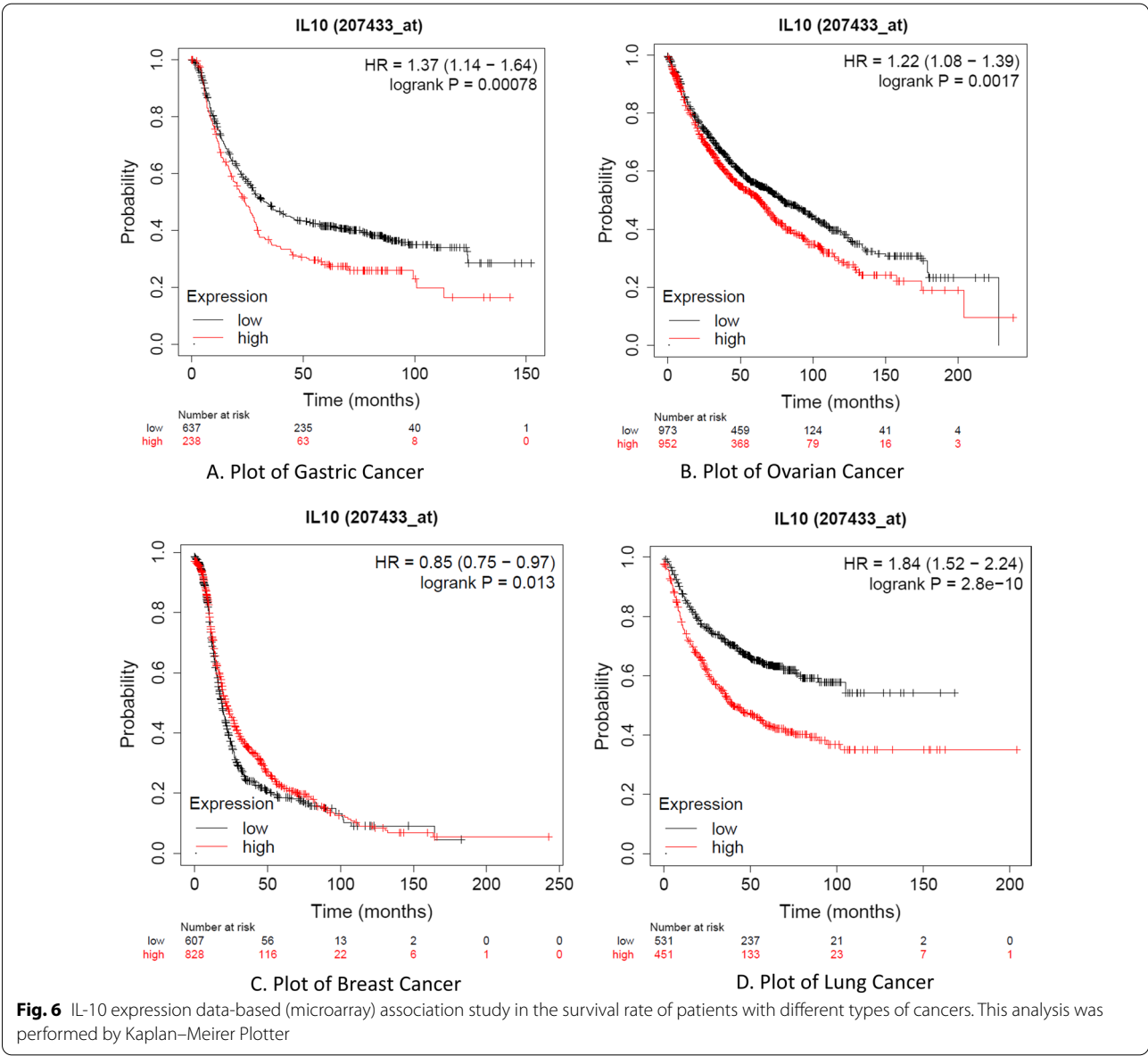
Table 5 Effects of amino acid changes on IL-10 protein from Project Hope

Amino acid change	Structure	Domain	Conservation	Loss of hydrogen bond or other
R42G	The mutation introduces glycine at this position. Glycine are very flexible and can disrupt the required rigidity of the protein at this position	The mutated residue is located in a domain that is important for binding of other molecules. Mutation of the residue might disrupt this function	Highly conserved region	Loss of interaction, Loss of H bond
R45Q		The mutated residue is located in a domain that is important for binding of other molecules. Mutation of the residue might disrupt this function	Near highly conserved region	Charged lost: loss of interaction, size differs: loss of interaction
F48L		The mutated residue is located in a domain that is important for binding of other molecules. Mutation of the residue might disrupt this function	Highly conserved region	
E72G	May disrupt the required rigidity of the protein structure	None	Highly conserved region	Loss of H-bond or disrupt correct folding
M95T	The wild type residue is located in a region annotated in UniProt to form an alpha- helix. The mutation converts the wild-type residue in a residue that does not prefer alpha helices as secondary structure	None	Highly conserved region	Hydrophobic interaction lost
A98D	The wild type residue is located in a region annotated in UniProt to form an alpha- helix. The mutation converts the wild-type residue in a residue that does not prefer alpha helices as secondary structure	None	Highly Conserved	Hydrophobic interaction lost
R125S	The mutated residue is located in a domain that is important for binding of other molecules. Mutation of the residue might disrupt this function		Highly conserved	Loss of hydrogen bond or disrupt correct folding
Y155C	The mutated residue is located in a domain that is important for binding of other molecules. Mutation of the residue might disrupt this function		Highly conserved	Loss of hydrogen bond or disrupt correct folding
I168T		None	Highly conserved	Hydrophobic interaction lost

Fig. 5 Protein–protein interaction network of IL-10 protein using STRING
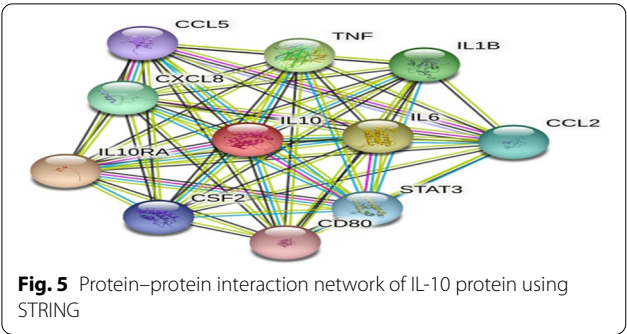
have structural and functional consequences that may lead to enhanced immune response. This observation suggests that the nine nsSNPs could prioritize diseases like Crohn's disease, allergy, autoimmunity, and other immune-related disorders.

The data obtained from STRING analysis reveal that the IL-10 protein has many vital functions: it inhibits the synthesis of several cytokines, including INF-gamma, IL-2, and IL-3, TNF GN-CSF produced by activated macrophages and by helper T-cells (Fig. 5). It also regulates the growth and differentiation of various cells such as B cells, NK cells, cytotoxic and helper T cells, and other immune cells [2]. Several studies revealed that low level of IL-10 aggravates autoimmunity pathology and disease severity in

Fig. 6 IL-10 expression data-based (microarray) association study in the survival rate of patients with different types of cancers. This analysis was performed by Kaplan–Meirer Plotter

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patients with multiple sclerosis (MS), juvenile onset arthritis, rheumatoid arthritis (RA), severe asthma, and systemic lupus erythematosus (SLE) [25, 60–64]. The abnormal function of IL-10 caused by the identified nsSNPs might enhance the severity of the mentioned diseases.

IL-10 also shows tumor-promoting and tumor-inhibiting properties. Elevated levels of *IL-10* are associated with increased tumor growth with poor prognosis and drug resistance. Again, elevated *IL-10* expression down regulates class-I and other cytokines that results in control metastasis and inhibits tumorigenesis. Previous studies showed that IL-10 might contribute to gastric cancer pathogenesis [65]. Similarly, a high expression level of *IL-10* was reported in ovarian cancer and found to inhibit ovarian cancer cell growth via downregulation of inflammatory cytokine production [66]. The dual effects of IL-10 could be the result of the concentration ranges of this protein. Through this study, elevated *IL-10* gene expression has been shown to govern positive significance on overall survival of gastric and ovarian cancer patients (Fig. 6). Any kind of deregulation caused by SNPs in *IL-10* gene might create drastic effects on the survival rate of gastric and ovarian cancer patients. It has been found in many studies that a low level of *IL-10* expression is associated with the development of human cervical, sporadic colon, and prostate cancer [67–69]. It cannot be neglected that a functionally defective version of IL-10 protein could reproduce similar types of phenotype as observed in the lowly expressed IL-10 patients. However, further studies are warranted in order to verify the correlation between defective IL-10 protein and the development of different types of cancers.

Conclusion

In this study we identified nine putative deleterious nsSNPs of *IL-10* by using multiple in silico tools. We believe identification of these nsSNPs should aid in the cost-effective and fast screening method to diagnose diseases that are related to *IL-10* expression. Additionally, it will greatly ease the approach of experimental designing for future laboratory-based research.

Abbreviations
IL-10: Interleukin-10; SNP: Single nucleotide polymorphism; TOPMed: Trans Omics for Precision Medicine; nsSNP: Non-synonymous SNP; Tregs: Regulatory T-cells; MDSCs: Myeloid derived suppressor cells; NCBI: National Center for Biotechnology Information; SIFT: Sorting Intolerant from Tolerant; SVM: Support-vector machine; PROVEAN: Protein Variation Effect Analyzer; PhD-SNP: Predictor of human Deleterious Single Nucleotide Polymorphism; PolyPhen- 2: Polymorphism Phenotyping v2; PANTHER: Protein analysis through evolutionary relationship; ΔΔG: Delta Delta G; INPS-MD: Impact of Non-synonymous mutations on Protein Stability-Multi Dimension; GEO: Gene Expression Omnibus; EGA: European Genome Phenome Atlas; TCGA: Cancer Genome Atlas; OS: Overall survival; HR: Hazard ratio; TI: Tolerant index.

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