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Research Article

Comparative Analysis of Commercial CCL21 and CCL21/IL1β Recombinant Proteins by *in silico* Tools

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Abstract

One of the newest diagnostic methods and treatment of cancer is to design new drugs. It is now possible to design a drug with desired properties in theory and evaluate its therapeutic effects through bioinformatics tools. Among the studied drugs, those based on cytokine genes, which increase the body's immunity against cancer, are of great interest. Cytokines are small proteins that play an essential role in cell signaling and can affect the function and behavior of surrounding cells. CCL21 chemokine is one of the cytokines that possess antitumor properties has the potential for chemoattraction of T lymphocytes and dendritic cells. Interleukin 1 beta (IL1β) is a cytokine involving different cellular activities such as the activation of neutrophils, B-Cells, and T-Cells. In the present study, we designed a drug-based cytokine gene to activate T cells and B cells by inserting defined CCL21 epitope and IL1β peptide sequences into a protein construct. Molecular dynamics simulation was performed in Linux space using Gromex software. Results of RMSD, RMSF, and the radius of gyration obtained from the simulation showed the stability of both proteins, which indicated that there are no significant conformational differences between the commercial CCL21 and recombinant form. The interaction of synthetic construct and human CCL21 with the CCR7 receptor was also investigated by HADDOCK software. Obtained results showed no differences between these proteins, and recombinant protein has the same structural and conformational characteristics as human commercial CCL21.

***Keywords*:** Cytokine, Chemokine, CCL21, Docking, Molecular Dynamics Simulation

Introduction

Nowadays, immunotherapy is a well-known method for understanding the problems related to the side effects of chemical drugs, analyzing the functional immune system during treatment, thus preventing tumor production. Among the drugs being studied for immunotherapy are cytokine-based drugs that increase immunity against cancer. Cytokines are small proteins that play an important role in cell signaling and affect surrounding cells' function and behavior. Cytokines include interferons, tumor necrosis factors, lymphokines, chemokines, and interleukins secreted by immune cells, mast cells, and various stromal cells (Akhter, Wu, Memon, & Mohsin, 2015).

Chemokines are a family of cytokines involved in the direct migration of leukocytes and activation of inflammatory stimuli. Chemokines and their receptors play a vital role in the growth, survival, or death of tumor cells as well as their metastasis (Jorgensen et al., 2019; Moore, 2001). Chemokine C-C motif ligand 21 (CCL21) is a cytokine that binds specifically to the CCR7 chemokine receptor, which has antitumor properties and can predict tumorigenesis in cancer (Madej et al., 2013; McHugh, 2019). CCL21/CCR7 has essential roles in immune cell and lymph-node homing, peripheral tolerance, development and function of T regulatory cells, and lymphoid neogenesis (Joutoku et al., 2019; Zhao et al., 2014). Increased CD8+ T cells can reduce the progression of viral diseases such as HIV and COVID-19. High expression of these genes acts as biomarkers in various diseases such as cancers and viral infections like HIV infection and pneumonia (Cyster, 1999; Gollmer et al., 2009; Jorgensen et al., 2019). CCL21 is known as a base for cancer immunotherapy since it can chemoattract T lymphocytes and DCs (Beemiller, Jacobelli, & Krummel, 2012). DCs receive tumor antigens and migrate to T-cell zones of lymphoid organs for particular antitumor T-cell activity (Zhao et al., 2014). Interleukin-1 beta (IL1β) belongs to the family of cytokines with severe proinflammation and, the *IL1β* gene encodes it in humans. IL1β is involved in various cellular

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activities such as activation of neutrophils, T and B lymphocytes, production of cytokines, antibodies, collagen, and fibroblast proliferation. (Van Damme et al., 1985). VQGEESNDK epitope is a part of IL-Iβ that acts as a potent adjuvant by binding to secretory protein sequences. This peptide sequence possesses all of the IL-Iβ adjuvant activity without any inflammatory response, such as induction of a fever response. (Boraschi, Tagliabue, & Miller, 2009).

Molecular dynamic simulation (MD) is a computer simulation method used to understand the conformational changes in recombinant proteins due to mutations comparatively(Musiani et al., 2014). In addition, its dynamics information can be used to analyze the highly fluctuating and complex nature of protein dynamics (Gaieb & Morikis, 2017).

The project's goal was to use immunoinformatic methods based on drug design algorithms to simulate and produce a drug based upon cytokine genes. Therefore, due to the complexity of the discovery process and the efficiency of neural network techniques, in addition to molecular docking, neural network techniques with neurophase rules were used to design an efficient diagnostic model in the immune system.

Materials and Methods

Construction of Amino acid Cassette

The epitope of human CCL21 (Accession No: CAG29322.1) and epitope of human IL-1β (PDB ID: 4G6M_A) were designed as the principal part of the recombinant protein cassette. So, the best T cell epitope of CCL21, EAAAK sequence as beta-defensins linker, the epitope of human IL-1 beta, RRVR as sensitive foreign protease linker, the signal SEKDEL for effectual agglomeration of the plant recombinant protein in the endoplasmic reticulum (ER), RVLAEA sequence as HIV protease linker and 6xHis tags was possessed (Figure 1)

Prediction of T- and B-Cell Epitopes of the Recombinant Protein

T-cell and B-cell epitopes of this recombinant protein were identified by BepiPred 2.0, BCpreds, ABCpreds, SVMTrip, and MAPPP online servers (Table 1 and 2). These bioinformatic tools have been used to prognosticate antigenic epitopes presented on the T-cell and B-cell surface by major histocompatibility complex class I and II molecules (MHC I, MHCII) (O'Donnell, Rubinsteyn, & Laserson, 2020). TAPPred server was used to verify the recombinant protein binding affinity of peptides toward the TAP transporter. This online service is based on cascade SVM and uses the amino acids sequence and properties.

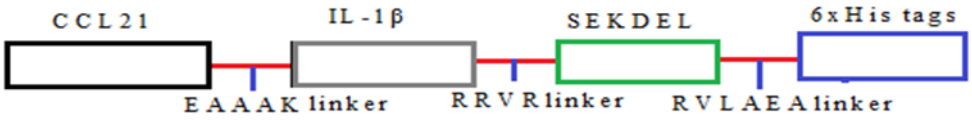


Figure 1. A) Schematic representation of CCL21/IL1β protein construct.

Table 1. Prediction of T- cell epitopes of human CCL21 (PDB ID: 2L4N). A covering score over 90% and IC50 below 50 were the best binder epitopes

MHCI			MHCII		
Epitope peptide	**allel HLA**	**score**	**Epitope peptide**	**allel HLA**	**IC50**
IPAKVVRSY	HLA-B*35:01	99.4%	LWVQQLMQH	HLA-DRB4*01:01	15.60
LPRKRSQAEL	HLA-B*07:02	95.7%	ELWVQQLMQ	HLA-DRB4*01:01	17.10
LCADPKELW	HLA-B*58:01	95.4%	PKELWVQQ	HLA-DRB4*01:01	18.50
LCADPKELW	HLA-B*57:01	93.4%	AKVVRSYRK	HLA-DRB4*01:01	19.20
IPAKVVRSY	HLA-B*53:01	90.3%	QQLMQHLDK	HLA-DRB4*01:01	21.40

Table 2. Prediction of T-cell epitopes of CCL21/IL1β protein.

MHCI			MHCII		
Epitope peptide	**allel HLA**	**score**	**Epitope peptide**	**allel HLA**	**IC50**
IPAKVVRSY	HLA-B*35:01	99.4%	SGTNDAEDCCLSVTQ	HLA-DRB1*08:02, HLA-DRB5*01	14.20
KELWVQQLM	HLA-B*40:01	93%	CAPPDQPWVERIIQR	HLA-DRB1*04:01	16.20

A covering score over 90% w and IC50 below 50 were the best binder epitopes of MHCI and MHCII-related HLAs, respectively.

Prediction of Physicochemical Characterization of Recombinant Protein

The SOLpro server measured the solubility of recombinant protein. Furthermore, ProtParam online server was used to identify various physicochemical parameters, including amino acid composition, pI, aliphatic index (II), instability index, in vivo and in vitro half-life, molecular weight (MW), and grand average of hydropathicity (GRAVY). The SignalP 5.0 server predicts the presence of signal peptides and the location of their cleavage sites in recombinant and commercial proteins. Localization of protein was analyzed by DeepLoc online server. The allergenicity of this recombinant protein was assessed by the AlgPred web server, which was used to show the post-translational modifications of CCL21/IL1β. NetOGlyc 4.0 Server was used to show the O-glycosylation and NetNGlyc 1.0 Server to show the N-glycosylation site of this recombinant protein. NetPhos 3.1 Server was used to find the phosphorylation sites of the protein (Safavi et al., 2019).

Molecular Dynamic Simulation and the Prediction of the Stability and Flexibility of the Recombinant Protein

PSIPRED webserver was used for computational modeling and getting the PDB file of the CCl21/IL-1β construct. Molecular dynamic (MD) simulations were utilized using GROMACS-4.5 and GROMOS96 (ffG45a3) force fields to assess the conformational changes of the protein. In order to neutralize the system in terms of charge, 5 counter Cl- ions for CCl21 and 7 counter Cl- ions for CCl21/IL-1β simulation were added to the solvated system. Pressure and temperature were kept at 1 bar and 300 K, and the system ran for 20 nanoseconds. The root-mean-square deviation (RMSD), the root-mean-square fluctuation (RMSF), and the radius of gyration of C-alpha atoms were calculated and analyzed using the Grace software. Also, the PDB files of the two proteins were aligned using the Pymol software.

Homology Modeling Structure Using *in silico* Tools

Since the 3D structure of this recombinant protein is not available, the comparative modeling method was used to create its three-dimensional

structure. Comparative modeling is one of the best methods for obtaining the three-dimensional structure of a target protein, where three-dimensional structures that have very similar sequences to the target sequence are used as a model. One of the online software used for comparative modeling is the Swiss Model. We also used the 1.7.0 version of Pymol software for modeling our target recombinant protein.

Molecular Docking of Cytokine Ligands and CCR7 Receptor

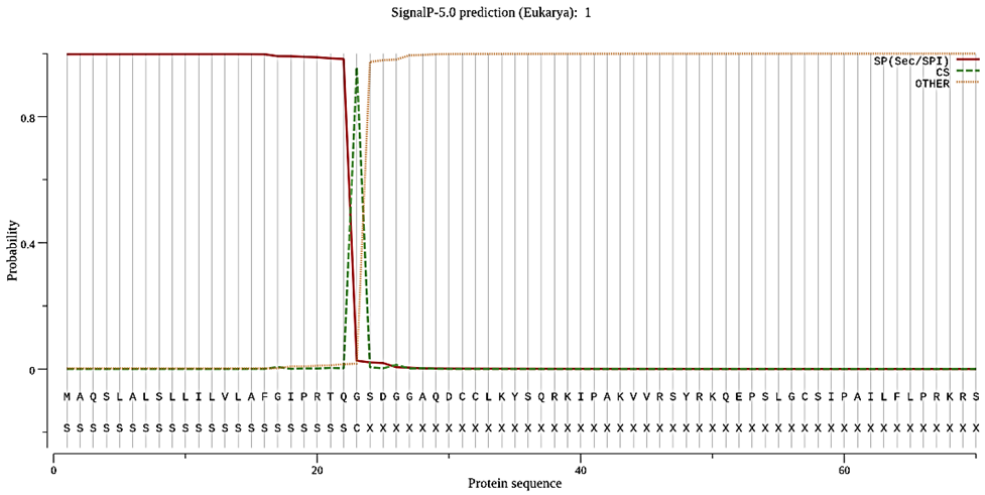
HADDOCK software was used to compare the interaction of CCR7 - CCl21/IL-1β and CCR7 - CCL21. HADDOCK software can use biochemical and biophysical information obtained from laboratory methods to predict interaction. This program performs protein-protein docking in a completely flexible manner. Amino acids obtained from these methods are defined as ambiguous interaction constraints (AIRs). AIR is then used to perform molecular docking. This energy is the sum of electrostatic energies, Van der Waals. In this method, amino acids that more than 50% of their external surfaces are exposed to water are considered active amino acids. The active amino acids of the cytokine ligands were identified according to mentioned protocol. Also, extracellular amino acids of CCR7 protein were considered as active amino acids.

Results and Discussion

Evaluation of the Physicochemical Properties of Recombinant Protein

Previously mentioned servers predicted the physicochemical analysis of the recombinant protein. The presence of signal peptides in commercial CCL21 and CCL21/IL1β recombinant proteinS was predicted by The SignalP 5.0 server. As shown in figure 2, the presence of signal peptides in 23 primary amino acids and the location of their cleavage sites in the 24th amino acid of commercial CCl21 was detected. However, there is no signal peptides in 65 primary amino acids of CCL21/IL1β recombinant protein were reported; obtained results was based on the fact that 23 primary amino acids as signal peptide were removed from CCL21/IL1β construct and the sequences of the signal SEKDEL for effectual agglomeration of the plant recombinant protein in the endoplasmic reticulum was replaced at the end of the construct. The molecular weight (Mw) of both native and recombinant proteins was estimated as 14'884.5504 Da and 14'893.834 Da. Isoelectric point values (pI) were 9.377 and 9.231, respectively, and both proteins' solubility was about 0.84.

Furthermore, the instability index of native protein was 37.8, and recombinant protein was 38.03, which confirms the stability of these proteins. In both proteins, the computed half-life in the mammalian, yeast, and E. coli cells was more than 30 h, 20 h, and 10 h, respectively. Aliphatic index and GRAVY were determined to be about 49 and -1.2, respectively, which shows that both proteins possess hydrophilic properties. As shown in Figure 3 and Table 3, this protein is localized in the nucleus. The allergenicity of recombinant protein was assessed, and the prediction accuracy was 94% at the -0.4 threshold. Therefore, unlike commercial CCL21 protein, recombinant protein has no allergenic effects.



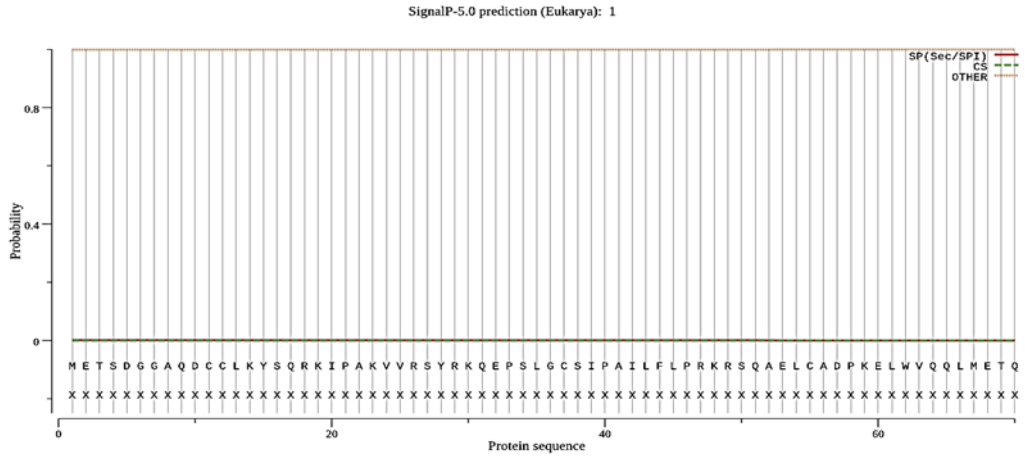


Figure 2. Prediction of the presence of signal peptides in commercial CCl21 and CCL21/IL1β recombinant protein by the SignalP 5.0 server; A) presence of signal peptides in 23 primary amino acids the location of their cleavage sites in 24th amino acid of commercial CCl21 were detected. B) as shown in this figure, no signal peptides in 65 primary amino acids of CCL21/IL1β recombinant protein.

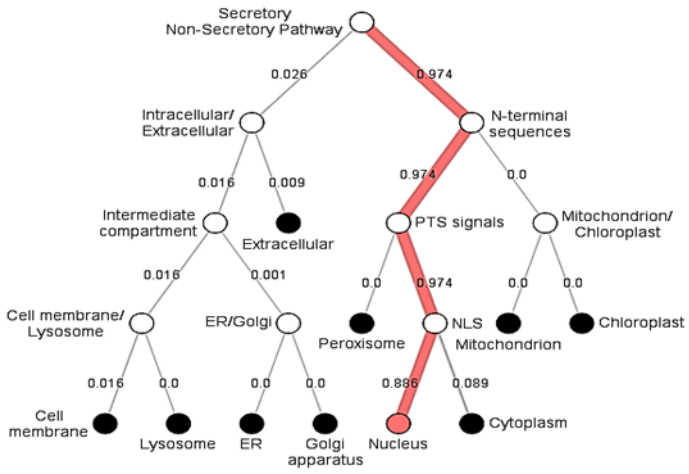


Figure 3. Schematic representation of subcellular localizations CCL21/IL1β protein construct. This soluble and extracellular protein that localized in the nucleus with a Likelihood of 89.9 percent.

Table 3. Prediction of subcellular localization of CCL21/ Il1β constructs in human cells.

Localization	Nucleus	Cytoplasm	Cell membrane	Extracellular	Mitochondrion	Endoplasmic reticulum	Golgi apparatus	Plastid	Peroxisome	Lysosome/ Vacuole
Likelihood	0.8918	0.093	0.015	0.0079	0.0009	0.0005	0.0005	0.0002	0.0001	0

Type	Soluble	Membrane
Likelihood	0.8434	0.1566

Prediction of T- cell and B-cell Epitopes of the Recombinant Protein

T-cell and B-cell epitopes of recombinant protein were identified. All T cell epitopes of human CCL21 and CCL21/IL1β were illustrated in

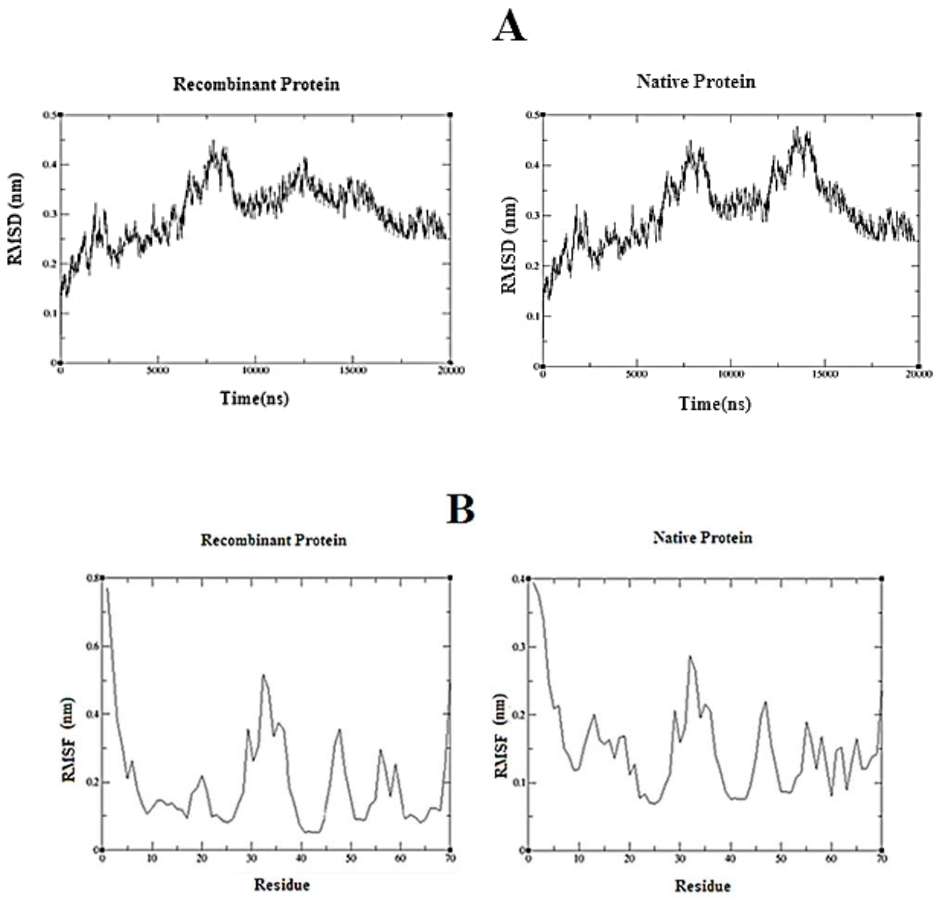
Tables 1 and 2. There were no B cell epitopes predicted in human CCL21, but the VQGESNDK sequence of IL1β was predicted as a B cell epitope.

Protein Structure Conformational Flexibility and Stability Analysis

In this project, we used MD simulations to compare the conformational changes of native and recombinant proteins. Multiple were analyzed throughout the simulation project, chiefly root mean square deviation (RMSD), root mean square fluctuations (RMSF), and the radius of gyration of the proteins with the time-dependent function of MD. Obtained results proved that the dynamic motions of the two proteins are very similar.

RMSD values of CCl21 commercial antigen, native protein, CCL21/IL-1β recombinant antigen, and mutant proteins were analyzed to identify the effect of mutations on recombinant protein structure. We calculated RMSD for protein backbones and found RMSD values from the mutant structures to be quite stable, like the native protein.

The CCl21 antigen and recombinant protein were stabilized at an RMSD value of around 5 Å, demonstrating that the mutations did not destabilize the protein structure (Figure 4A).



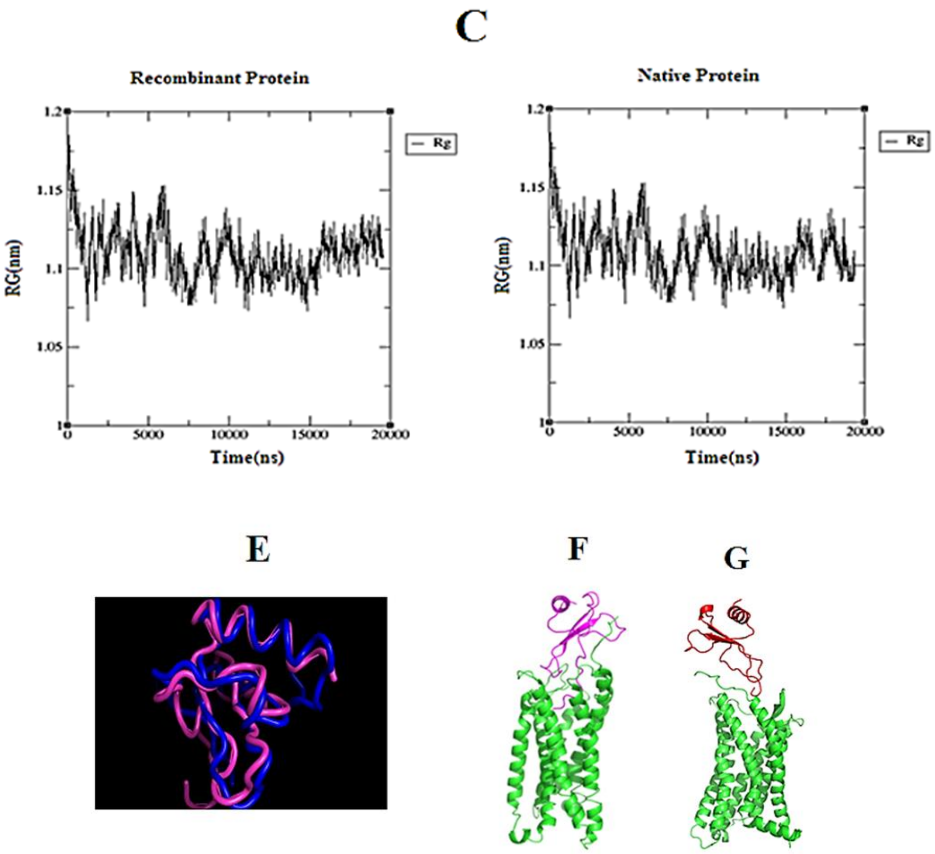


Figure 4. Protein structure conformational flexibility and stability analysis of CCl21 constructs, as the recombinant protein, and commercial CCl21, as the native protein, for 20 ns MD simulations. A) RMSD values during MD simulations of the recombinant protein and native protein structure. CCl21 antigen and recombinant protein were stabilized at an RMSD value of around 5 Å. B) Calculated average RMSF for Cα atoms of the recombinant and native protein structure, residues located between Positions 30 and 60, residue fluctuations for CCL21/IL-1β were similar to CCl21 commercial antigen and fairly low C) Radius of Gyration for the recombinant and native protein. The results of the radius of gyration indicated that CCl21/IL-1β and CCl21 commercial antigen have the minimum compactness of their structures with 11.8 Å value. E) Visualization of the native (blue) and mutant (red) PDB files, aligned with PyMol software to show the same structure and conformational characteristics of these two proteins. F) The molecular docking of the complex of CCR7 and CCl21/IL-1β. G) The molecular docking of the complex of CCR7 and human CCl21.

We also analyzed the RMSF fluctuations of each residue to specify the effect of mutations on protein residues. As Figure 4B demonstrates for residues located between positions 30 and 60, residue fluctuations for CCL21/IL-1β were similar to CCl21 commercial antigen and fairly low.

The results of the radius of gyration indicated that CCl21/IL-1β and CCl21 commercial antigen have the minimum compactness of their structures with 11.8 Å value. These data show that CCl21 mutations did not cause structural destabilizing effects, and no significant alterations were found for either protein's compactness during the simulation (Figure 4C).

Visualization Analysis of Native and Recombinant Protein

The PDB files of two proteins were aligned together by PyMol software to compare the conformational differences of the native and recombinant proteins (Figure 4E). Put together. Our findings verify that these proteins have the same structural and conformational characteristics.

Comparative Analysis of Molecular Docking of these Two Proteins with CCR7 Receptor

This project aimed to investigate and comparative analysis of interactions between these proteins and CCR7 receptors. As shown in Table 3, molecular docking of human CCL21 was determined, and the best cluster had a score of -27, with a size of 34 complexes. In addition, Z-score was equal to -2.3. Molecular docking of CCl21/IL-1β was also determined. The score of the best

Table 4. The best cluster of molecular docking result

protein	HADDOCK score	Cluster size	**Z-Score**
human CCL21	-27	34	**-2.3**
CCL21/IL1β	-30	38	**-2.8**

cluster was -30, with a size of 38 complexes. In addition, Z-score was equal to -2.8 (Table 4). Results obtained from molecular docking of these two proteins were so similar, and as mentioned before, there were interactions between CCL21/IL1β ligands and CCR7 receptors as human CCL21. The image of the complex of CCR7 - CCl21/IL-1β and CCR7 -CCl21 has been shown in Figures 4 F & 4 G.

Conclusion

The findings of this experiment confirmed that the recombinant protein and commercial CCL21 have the same structural and conformational characteristics. Therefore, this recombinant protein maybe has the same function as commercial CCl21, like anti-metastatic and cytotoxicity effects on cancer cell lines. Also, it has a chemotactic response on lymphocyte cells and is a potential treatment option in cancer immunotherapy. We have achieved our predetermined goals: to produce the recombinant protein in different expression hosts like yeast and plant to improve the production of CCL21 recombinant protein. Other diagnostic tests should be performed before the clinical application and commercialization of this protein.

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