Research Article

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In silico study of lactoferrin as an anti-cancer, anti-viral, and anti-bacterial bioactive compound and disease diagnostic marker.

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ABSTRACT

Studying the interaction of proteins and genes by current computational methods is effective in new drug design. Considering the special role of lactoferrin (Lf) identifying its action mechanism is a new gate to the targeted treatment of some diseases. This study aimed to investigate the molecular interaction of Lf with virus spike, the bacterial cell binding receptor, and the proteins involved in apoptosis by in silico research. The docking results showed that the amino acids involved in the interaction between the spike virus of COVID-19 with the ACE2 receptor were similar to the amino acids in the interaction between the virus spike and Lf, and the binding between Lf and the viral spike was more inclined. The N-terminus of Lf interacted with the N-terminus region of the CD14, which contains the binding sites of bacterial lipopolysaccharide to the cell. Lf can compete for binding with bacterial lipopolysaccharides. The results related to the docking of TP53, BAX, and BAK1 gene promoters and Lf showed that Lf with one or more amino acids that are responsible for binding to DNA interacted with TP53 and BAX genes with higher binding affinity. In all protein and protein-gene interactions, the N-lobe region of Lf, which is its functional region, is involved in these interactions, and the anticancer, antibacterial, and antiviral, roles of Lf are related to this region. Lf is important as a medicinal supplement for treating COVID-19 and cancer.

Keywords : *Cancer, COVID-19, In silico, Lf, Lipopolysaccharide, promotor.*

INTRODUCTION

Lacroferrin (Lf) is a type of glycoprotein with a molecular weight of 80 kDa, belonging to the transferrin family. Lf is a relatively stable protein that can be active as a digested fragment even after passing through the intestinal tract. This fragment with a molecular mass of more than 20 kilodaltons contains the binding region to the protein receptor on the cell as well as the active anti-cancer, anti-viral, and anti-bacterial regions [1]. Lf receptors have been identified on the surface of different cells and some of them can bind to both human Lf and bovine Lf (bLf). Also, different types of cells express their specific Lf receptors [2]. When Lf is used as a "biological drug," it appears to be orally active, unlike many other therapeutic proteins. Since Lf and its derivatives are food-derived components and therefore non-toxic, they can be an interesting alternative to chemotherapy and current anti-cancer and other drugs [2]. Structurally, it is a simple polypeptide chain that is folded into 2 symmetrical lobes, N and C lobes. These two lobes are connected by a hinge region containing amino acids 333-343 with an α-helix bond (in human Lf), which gives the molecule high flexibility [3]. The polypeptide chain consists of amino acids 1-332 for the N-lobe and 344-703 for the C-lobe and consists of helix-sheet structures that create two domains for each lobe (domain I and II) [4]. Each lobe can combine with a metal atom by sharing a carbonate ion (CO_3^{2-}). Metals that form bonds include Fe^{+2} and Fe^{+3}, but their connection with Cu^{2+}, Mn^{2+}, and Zn^{2+} ions has also been seen. Due to the reversibility of Fe^{3+} binding, Lf can exist free of Fe or bound with $Fe^{3,2+}$, each of which will have a different three-dimensional shape

[3]. The N-terminal region of Lf is highly basic and contains positively charged Arg and Lys amino acids. Interestingly, the natural human Lf protein begins with the sequence Gly-Arg-Arg-Arg-Arg, which resembles a nuclear localization sequence (for intranuclear activity) [5]. The surface of the Lf molecule has several regions with a high concentration of positive charge, which is responsible for one of the characteristics that distinguish Lf from other transferrins and is responsible for some unique properties [6]. The most significant positive charge region includes the N-terminal of the polypeptide chain, which separates from the protein surface, and the C-terminal adjacent to helix 1, where residues 27-30 have the RKVR sequence [7]. This region provides a site for the binding of heparin and glycosaminoglycans. Another major positive charge concentration is associated with the region between the two lobes that are joined by the helix. It is also attractive as a possible DNA binding region, both because of the charge and because of the cleft between the lobes, which could provide a docking site. The positive charge of Lf allows it to bind with negatively charged molecules on the surface of different cells of the immune system, and it has been suggested that this connection can be combined with signalling that leads to cellular responses such as activation, differentiation, and proliferation of immune cells. It has also been observed that Lf can activate various signalling by entering the cell nucleus through binding with DNA. The positive amino acids in Lf can interact with anionic molecules on some bacterial, viral, fungal, and parasitic surfaces and cause cell lysis [3,4]. Another isoform of the human Lf protein, known as delta-Lf (ΔLf), which lacks the first 25 amino acids of the natural protein, is expressed inside many cells, where it functions as a transcription factor [8]. ΔLf is a transcription factor whose expression is reduced in cancer. This gene is a marker of healthy tissue and the high expression level of its transcripts is associated with good prognosis in breast cancer. ΔLf is the result of using the alternating promoter of the hLf gene, which leads to the production of 2 isoforms with alternating N ends, including secreted Lf (isoform 1) and the nucleocytoplasmic counterpart ΔLf. ΔLf is a potent transcription factor that interacts in vivo with the ΔLf response element in the Skp1, BAX, DcpS, and SelH promoters [9]. It is known that the reduction or silencing of Lf or ΔLf genes in cells leads to an increase in malignant tumors. Instead, the proliferation of cancer cells is blocked following the reconstruction of the Lf gene [10,11]. A region in the Lf gene promoter that is activated in response to infection has been identified. Lf gene expression is stimulated by bacterial membrane lipopolysaccharide and double-stranded RNA [12].

Studies have shown that cancer cells have a relatively high prevalence of genetic polymorphisms, gene mutations and promoter hypermethylation in the Lf gene [13,14]. Since the restoration of Lf expression successfully reduces the growth of tumors, the Lf gene can be investigated as a new target site for cancer gene therapy. Alternatively, Lf may be used as a therapeutic drug or inhibitory compound in cancer treatment [2]. Interestingly, many studies have shown that Lf can spontaneously enter various eukaryotic cells, where it can affect gene transcription [5]. Many studies showed the presence of Lf receptors on the surface of tumor cells. Heparan sulfate plays an important role in regulating the binding of Lf and lactoferricin B with receptors [15]. Several studies have shown that lactoferricin B or its derivatives selectively kill cancer cells without adversely affecting normal cells [16-18]. This action can be due to the subtle differences between the cell membrane of normal cells and the membrane of cancer cells. The most obvious difference in the cell membrane is the existence of a much more negative charge in cancer cells [2, 19]. The structure of the cationic peptide of lactoferricin may facilitate its interaction with cell surface death receptors and intracellular apoptosis-related proteins [20]. Several mechanisms for the antitumor role of Lf, such as regulation of NK cell activity [21], cell cycle arrest [22], inhibition of VEGF-mediated angiogenesis [23], Iron absorption, and prevention of its oxidative effects [24] and increased apoptosis [22] have been identified. The proposed chemical inhibitory effects for Lf could be due to multiple functions of Lf, which include stimulation of immune response, regulation of cancer metabolizing enzymes and oxidant-antioxidant profile in target organs, and inhibition of angiogenesis. Regulation of immune system function by Lf may be a key factor in the mechanisms of action involved in cancer prevention [23, 25]. Lf can prevent cancer by suppressing viruses and bacterial infections. It is well established that 1 in 6 cancers are caused by infection, and suppression of infection plays an important role in cancer treatment after chemotherapy or bone marrow transplantation [26]. Antibacterial and antiviral properties can be very useful in preventing infection and cancer recurrence after chemotherapy treatments [24]. There is a lot of evidence that Lf has antibacterial activities in both in vivo and in vitro environments for gram-positive and gram-negative bacteria [27]. Lipopolysaccharide (LPS) is a non-protein component of the outer membrane of gram-negative organisms and a strong stimulus for cell inflammation. In addition, to complement activation, host stimulation factors are mediated mainly through CD14 which acts as a specific receptor for LPS [28]. Interference of the N terminus domain of Lf with the LBP domain, which mediates the binding of LPS to the mCD14 receptor, has been repeatedly shown to result in a significant reduction in the production of the major pro-inflammatory cytokines IL-1, IL-6, and tumor necrosis factor-alpha (TNF-α) [28, 29]. Antiviral activity of Lf has been reported against a wide range of viruses such as AIDS virus, enterovirus, and SARS-CoV-2. Lf by binding to

Journal of Biochemistry (ISSN 2995-6536)

the DNA and RNA of viruses, prevents their replication [30]. The antiviral activity of Lf includes preventing virus binding, inhibiting virus replication, and blocking cell-virus interaction [8, 31]. Considering that there is a close relationship between changes in the Lf gene and the increase in the incidence of cancer, therefore, the use of Lf as a new cancer marker may be used in the diagnosis and also in measuring the prognosis of cancer patients in different stages. Lf can be used as a biomarker for inflammatory diseases, as well as ulcerative colitis, Crohn's disease, inflamed knee joints, and rheumatoid arthritis [2, 32].

Lf can reduce the damage caused by oxidative stress due to its ability to chelate iron ions known as oxidative iron. High concentrations of iron can be toxic, iron can donate electrons to oxygen, and reactive oxygen species (ROS) are formed as superoxide anions and hydroxyl radicals [33]. If iron is out of balance, this cation can become toxic because it creates free radicals. Free radicals produce oxidative stress that leads to cellular redox imbalance. This imbalance may lead to oncogene stimulation [34].

Considering the importance of Lf protein and its important roles, especially its anti-cancer, anti-viral, anti-bacterial, and immune system modulating role, it seems that a series of bioinformatics studies and searching in the database to collect its mechanism involved in the mentioned roles can answer some of the researchers' questions regarding the pharmaceutical design of this protein or peptides derived from it. The aim of this study is to collect information about the mechanism of action, and the biochemical pathways of Lf function in cancer cells using experimental data and searching in the database, as well as molecular docking studies of this protein with some important proteins for the binding of bacteria and virus as well as the promoter of some important genes in the process of apoptosis.

MATERIAL AND METHODS

Bioinformatics databases

In this study, KEGG (https://www.genome.jp/kegg/pathway.html), PDB (http://www.wwpdb.org/), Cytoscape (https://www.ndexbio.org/viewer/networks), Signor (https://signor.uniroma2.it/covid), EPD (https://epd.expasy.org/epd) and UniProt (http://www.uniprot.org/) databases, as well as many literatures were used to extract information.

Molecular docking study between Lf protein and ACE2, mCD14 and S1 (COVID spike) proteins

First, the tertiary structures for Lf with the code 1bLf, the virus spike with the code 5GNB, the cell surface receptor ACE2 with the code 1R42, and mCD14 with the code 1WWL were extracted from the RCSB-PDB (Figures 5 A, B, 6 A, B, 7 A, B, and 8 A, B). Then, using the ClusPro webserver, the interaction of the three mentioned proteins with Lf was investigated, a blind docking methods and 3D structures of Lf and spike of virus, ACE2 receptor and mCD14 were uploaded on the webserver. The docking procedure was based on the probability of Lf binding to ACE2 or spike and also mCD14 . Then using PyMOL2.5 software, the interacting regions, and residues were visualized (The PyMOL Molecular Graphics System, Version 2.5 Schrödinger, LLC.). The amino acids involved in the interaction were also determined by the PDBsum (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/) website.

ClusPro

Molecular docking using the ClusPro (https://cluspro.bu.edu/login.php) webserver is widely used to investigate protein-protein interactions and is performed in three steps. First, they create a large number of conformations from the hard and inflexible state of the complex between proteins. In the second step, 1000 structures with the minimum energy are selected using the root-mean-square deviation (RMSD) based classification method, and among them, the largest groups that have possible complex models are identified. In the last stage, these models are optimized by minimizing unwanted energies in the system [36].

Molecular docking study between Lf protein and promoters of P53, BAX, and BAK1 genes

To investigate the interaction of promoters of P53, BAX, and BAK1 genes with Lf protein, their promoter sequences were extracted from the Eukaryotic Promoter Database. The three-dimensional structure of the promoter regions was modeled for docking. For this purpose, the promoter sequence along with their reverse sequence was uploaded on the 3dRNA/DNA website and their three-dimensional structure models were determined (http://biophy.hust.edu.cn/new/3dRNA). In this website, the DNA and RNA patterns determined by the experimental method available on the RSCB website were used as templates to construct the structure of DNA and RNA. To investigate the docking interaction between the determined structures of the promoter regions and Lf the pyDockDNA (https://model3dbio.csic.es/pydockdna) webserver was used [35]. In this web server, which is written using Python programming language, the scoring method is used based on electrostatic interactions and van der Waals interactions. The docking method used here was based on blind docking methods, as a result, the 3D structure of target DNA and protein was uploaded then the result was investigated with no prior information on preferred binding sites on promotors and Lf.

Journal of Biochemistry (ISSN 2995-6536)

RESULTS

Lf pathway in infection and coronavirus inflammation pathway

Lf can affect cellular DNA during stress and infection. Figure 1 shows the signal transduction pathway responsible for the activation of human nuclear factor-kappaB (NF-kappaB) by Lf during infection. Inflammation is an important part of the immune response. Unfortunately, infection with the COVID-19 and SARS-CoV-2 virus causes extensive and long-lasting inflammatory responses in some patients, known as a cytokine storm (Figure 2). Also, the human coronavirus induces apoptosis (Figure 3). Apoptosis is a programmed cell death process that is activated under cellular stress conditions and is characterized by a highly organized separation of cellular structures. Aberrant expression of several Human-CoV proteins such as S, E, M, N, 3a, 3b, 6, 7a, and 8a causes apoptosis in infected cells.

Figure 1

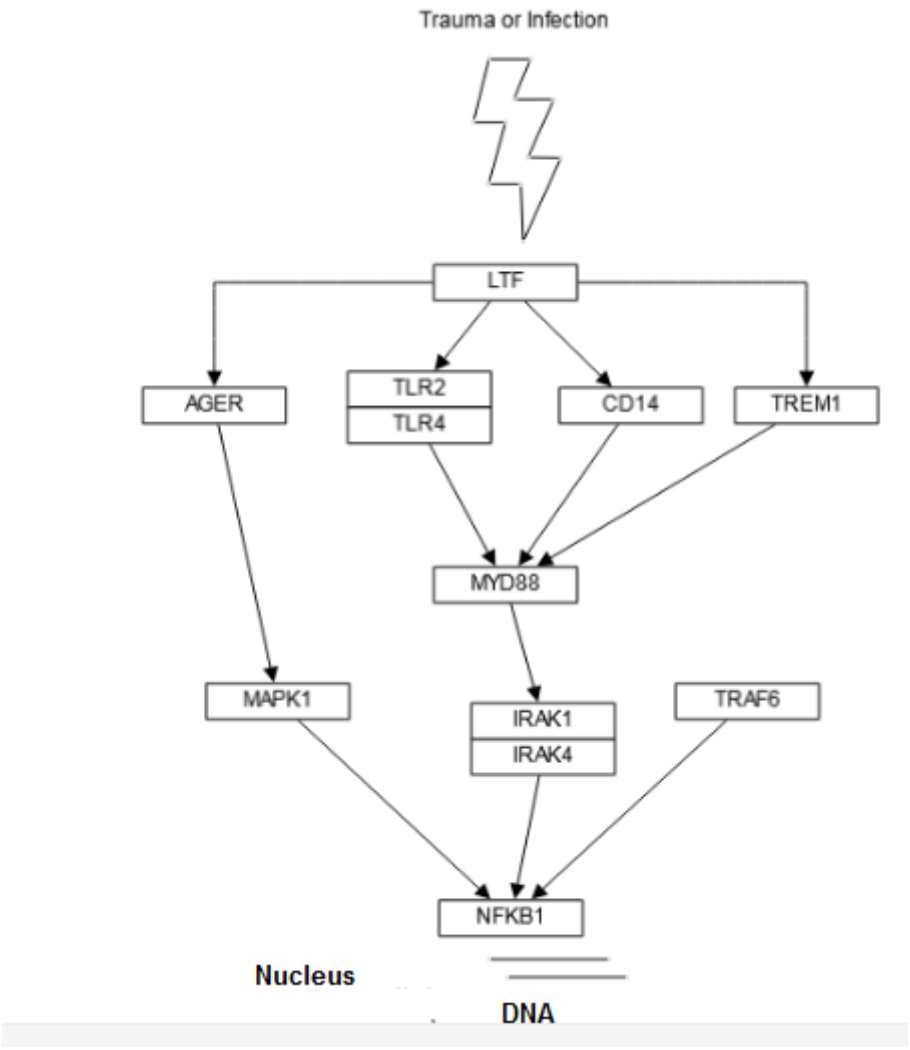

Figure 1. Image of Lf action pathway during infection and NF-kB induction (extracted from Cytoscape database, https://www.ndexbio.org/viewer/networks)

Journal of Biochemistry (ISSN 2995-6536)

Figure 2

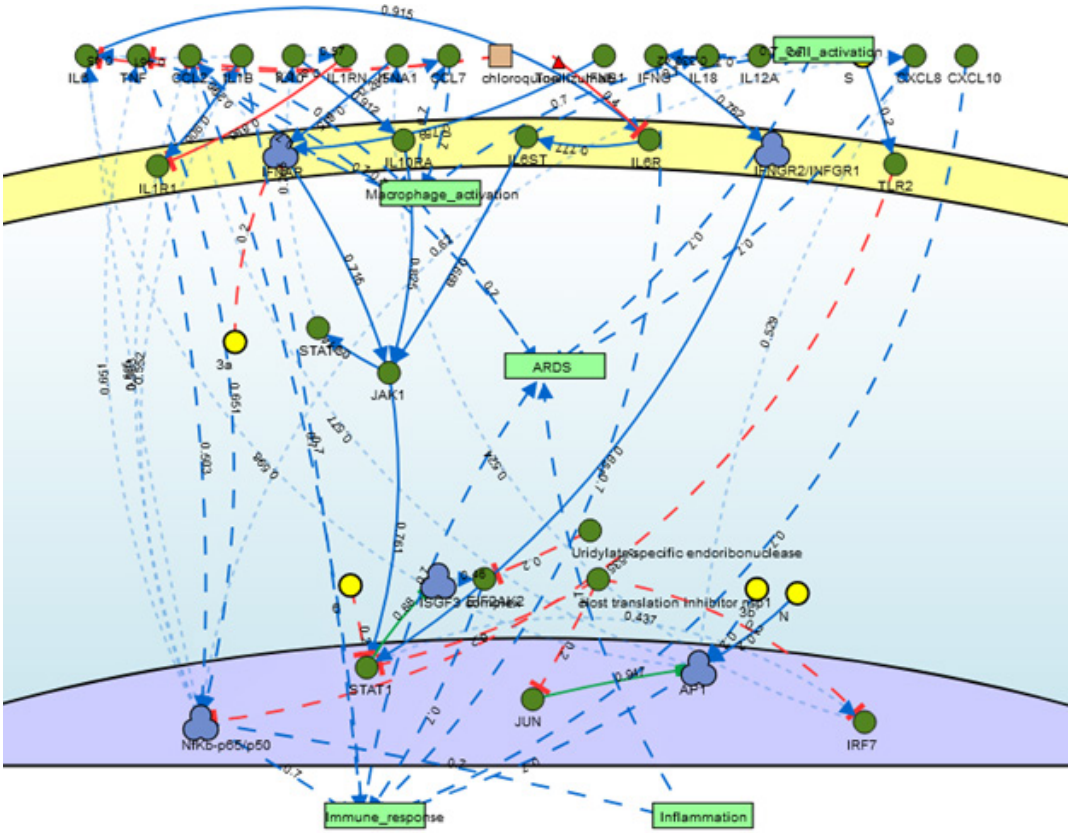

Figure 2. The effect of human corona virus in the induction of cytokine storm. The yellow circles show different proteins of the corona virus (https://signor.uniroma2.it/covid)

Figure 3

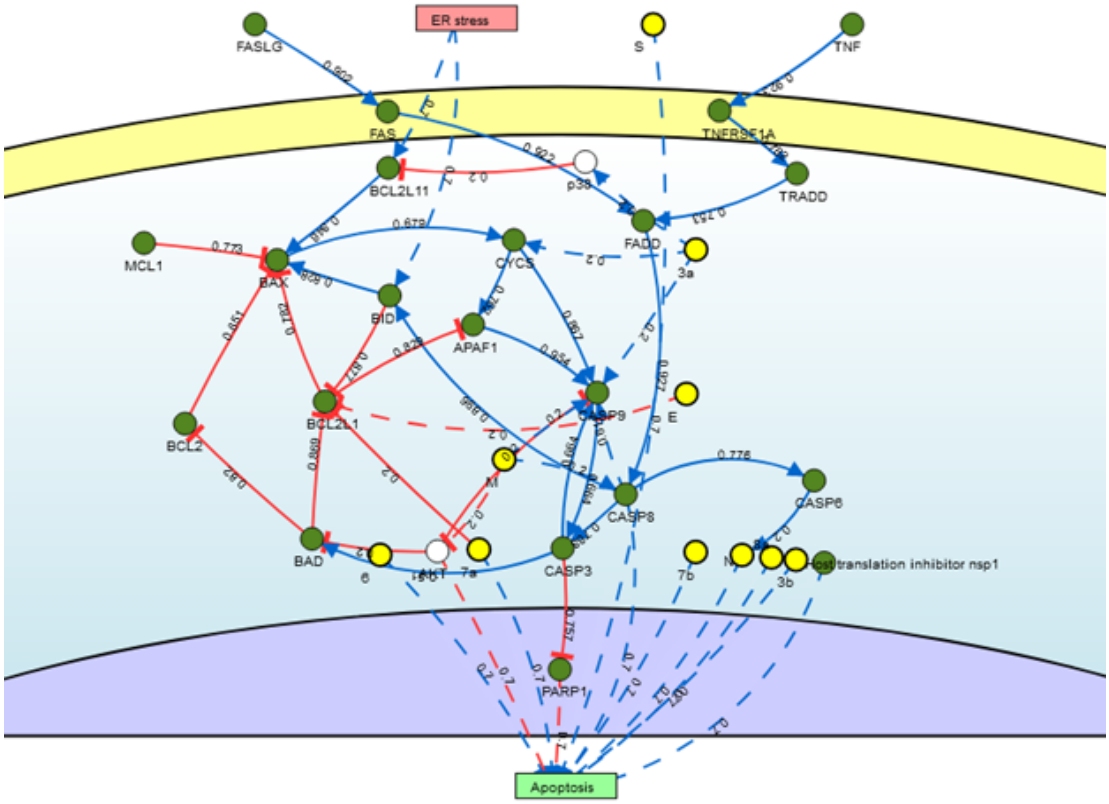

Figure 3. Effect of human coronavirus in induction of programmed cell death (apoptosis). The yellow circles show different proteins of the corona virus. (https://signor.uniroma2.it/covid)

Journal of Biochemistry (ISSN 2995-6536)

Lf binding to ACE2 and human covid spike

The extracellular part of the ACE2 receptor, which is important for the binding of the coronavirus, consists of two domains. The first domain, which is a zinc ion-containing metallopeptidase, consists of amino acids 19 to 611, and the second domain consists of amino acids 612 to 740. The subdomain at its N-terminus from amino acids 19 to 102, 290 to 397, and 417 to 430 is responsible for binding to the viral spike. Coronavirus binds to this receptor through its spike protein. In the C-terminal region of the S1 domain of the spike, there are various domains, including Core, SD-1, and insertion, which are important for attachment. In the study of protein-protein docking between Lf and the human ACE2 cell receptor, it was found that the binding mode models between Lf and ACE2 had the highest number of members, which shows that the best binding mode of these two macromolecules is more valid than the best binding mode of spike with ACE2 cell receptor in docking study. Also, the Weighted Score Lowest Energy showed that the binding between Lf and the viral spike has the highest tendency (**Table 1**). In the interaction between Lf and ACE2, it was found that Lf interacts with amino acids number 13 to 39 from the N-terminal region to different regions of the ACE2 receptor binding site for coronavirus spike protein, including amino acids number 135 to 171 and also amino acids in other regions (Figure 4 and Figure 5 D). The docking results disclosed that the amino acids involved in the interaction between the virus spike and Lf were similar to the amino acids involved in the interaction between the spike and the ACE2 receptor. The surface representation and hydrogen interactions between Lf and ACE2 protein in the best binding state are shown in Figure 5 C, D. The hydrogen interactions between Lf and the spike of the COVID-19 virus revealed that the amino acids of the N-terminal domain of Lf were bonded with the amino acids of the tip of the spike insertion region (the C-terminal domain). Residues H488, E505, T507, T508, V509, L510, H512, W515, R517, L521, Y528, and D529 from the spike insertion domain interacted with Lf. The C-terminal region and the domains of this region play a role in binding the virus to the cell receptor (Figure 6 C, D). To have confirmation on the docking results, docking between the ACE2 with spike protein was performed with the same procedure. Results indicated amino acids 390 to 579 of the C-terminus of the S1 spike region interacted with amino acids 89-563 of the N-terminus region of the ACE2 receptor (Figure 4 and 7 D). The results are greatly in agreement with experimental results as spike core and insertion loops interacted with the N-terminal of human ACE2 receptors (Figure 7 C, D).

Table 1. The results of docking analysis of the interaction between Lf protein and ACE2 receptor, as well as the S1 subunit of Spike protein of nCov-2019 virus

Proteins	Cluster Members	Weighted Score Center	Weighted Score Lowest Energy
ACE2-Lf	**152**	**-781.6**	**-949.6**
Lf-Spike	**125**	**-922.4**	**-959.1**
ACE2-Spike	**58**	**-798.8**	**-798.8**

Journal of Biochemistry (ISSN 2995-6536)

Figure 4

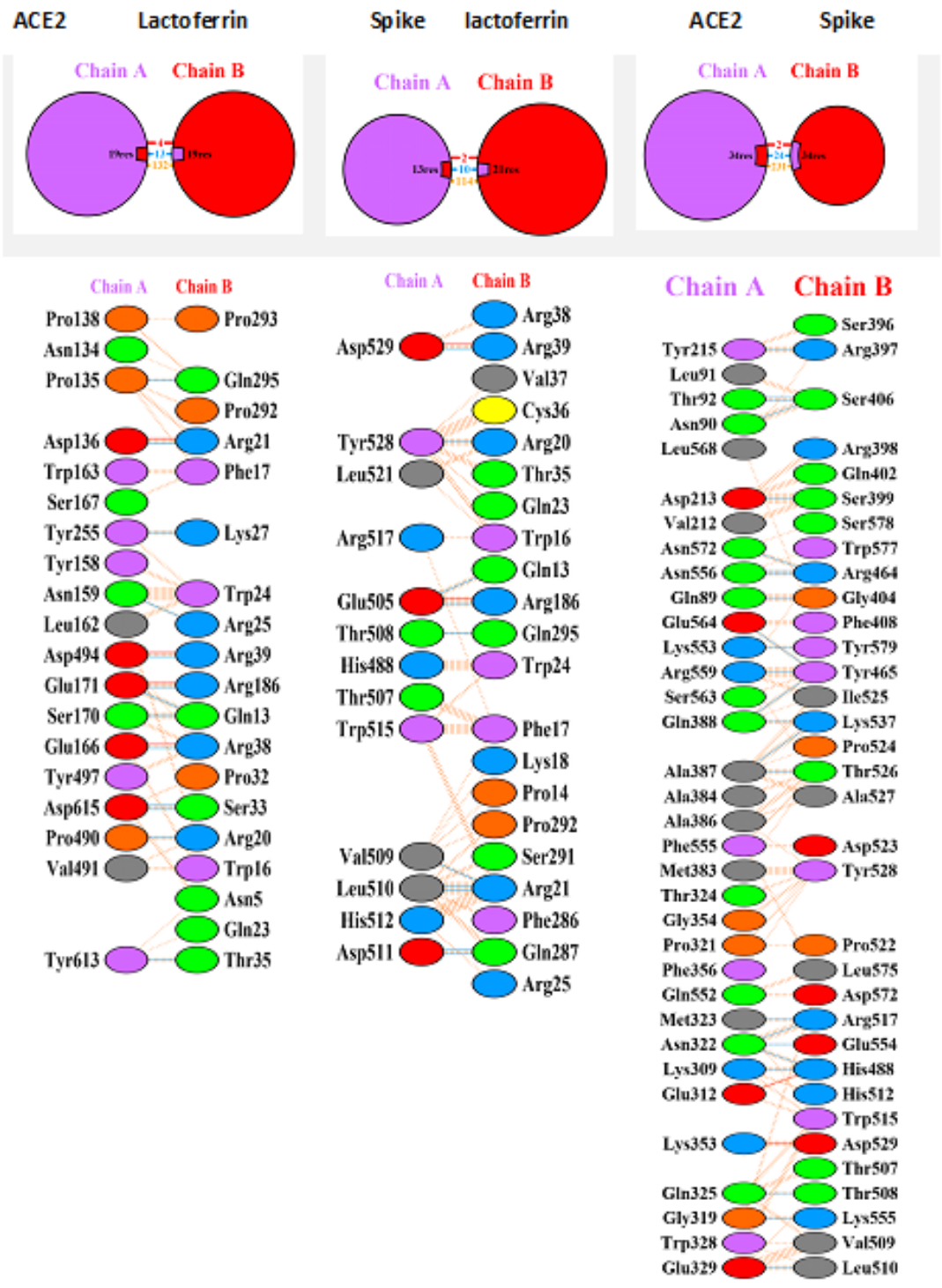

Figure 4. 2D representation of interactions between Lf proteins, ACE2 cell receptor and nCov-2019 spike obtained from Docking using Cluspro webserver prepared using PDBsum (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/) website.

Journal of Biochemistry (ISSN 2995-6536)

Figure 5

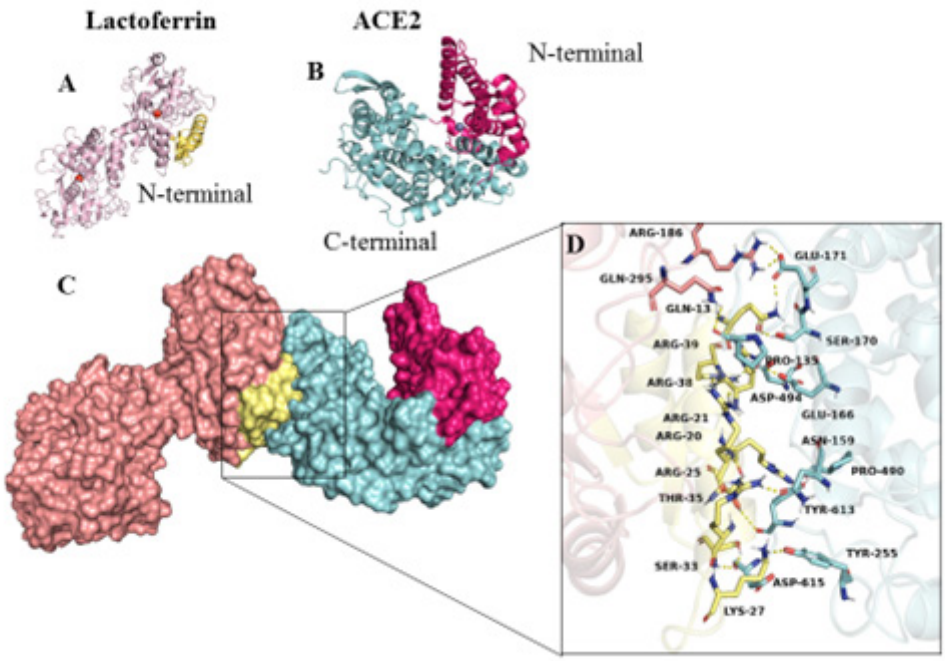

Figure 5. Lf and ACE2 interaction. A) Tertiary structure of Lf, B) Tertiary structure of ACE2, C) Surface representation of the interaction between Lf protein coded as bLf1 and cell receptor ACE2 coded as 1R42, and D) 3D representation of hydrogen bonds formed in the interaction obtained from molecular docking results using ClusPro webserver.

Figure 6

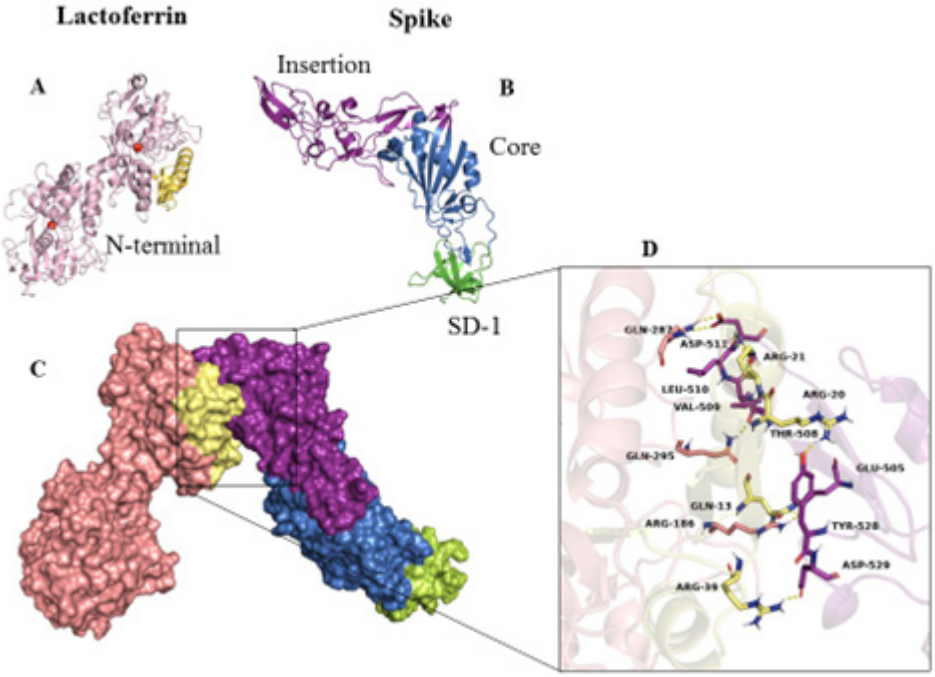

Figure 6. Lf and virus spike interaction. A) Tertiary structure of Lf, B) Tertiary structure of spike, C) Surface representation of the interaction between Lf protein coded as bLf1 and Viral spike with code 5GNB, and D) 3D representation of hydrogen bonds formed in the interaction obtained from molecular docking results using ClusPro webserver.

Journal of Biochemistry (ISSN 2995-6536)

Figure 7

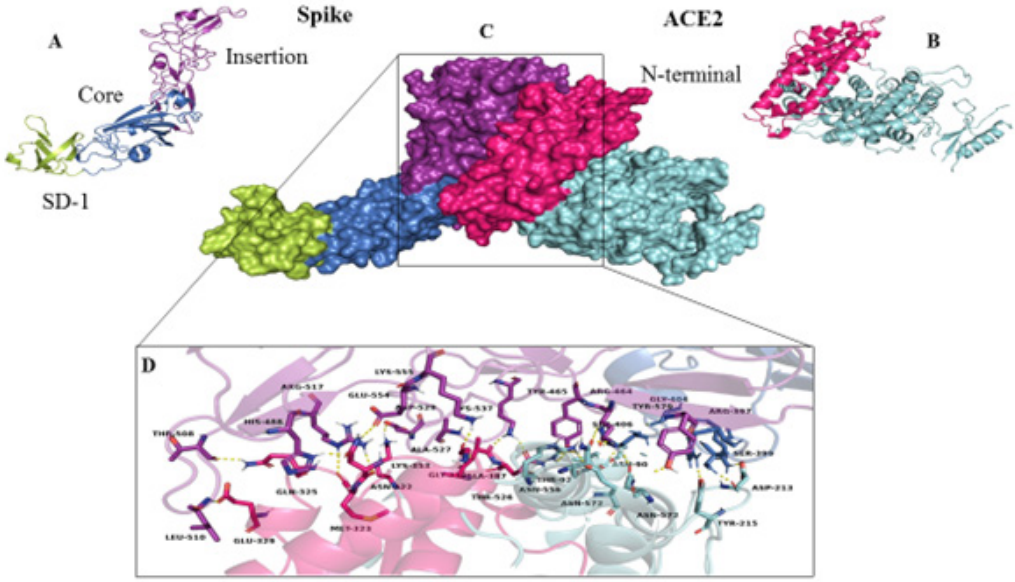

Figure 7. ACE2 and viral spike interaction. A) Tertiary structure of spike, B) Tertiary structure of ACE2, C) Surface representation of the interaction between ACE2 and Viral spike with code 5GNB, and D) 3D representation of hydrogen bonds formed in the interaction obtained from molecular docking results using ClusPro webserver.

Lf binding to CD14 receptor

The monomeric structure of the CD14 receptor has 13 beta chains as well as alpha and loop-like structures, and the N-terminus of this receptor has a main hydrophobic cavity that includes β1-3 and α1-4, which the most important amino acids of this region are 1-65 for the binding of bacterial lipopolysaccharide (Figure 8A). In the molecular docking between Lf and the cell receptor, Lf with its N-terminus, which has positively charged amino acids such as arginine and lysine, is attached to the N-terminus of the CD14 receptor, which includes amino acids number 9 to 69. The best binding mode showed the lowest energy of binding equal to -801.7 kJ/mol with a member number of 140, which indicates the model validation. However, the binding site of Lf is the same as the binding site of bacterial lipopolysaccharide to the receptor, but, visualization cleared that Lf attaches to the edge of the hydrophobic cavity as the Lf binding motif is positively charged by the presence of Agr of Lys residues (Figure 8 C, D).

Figure 8

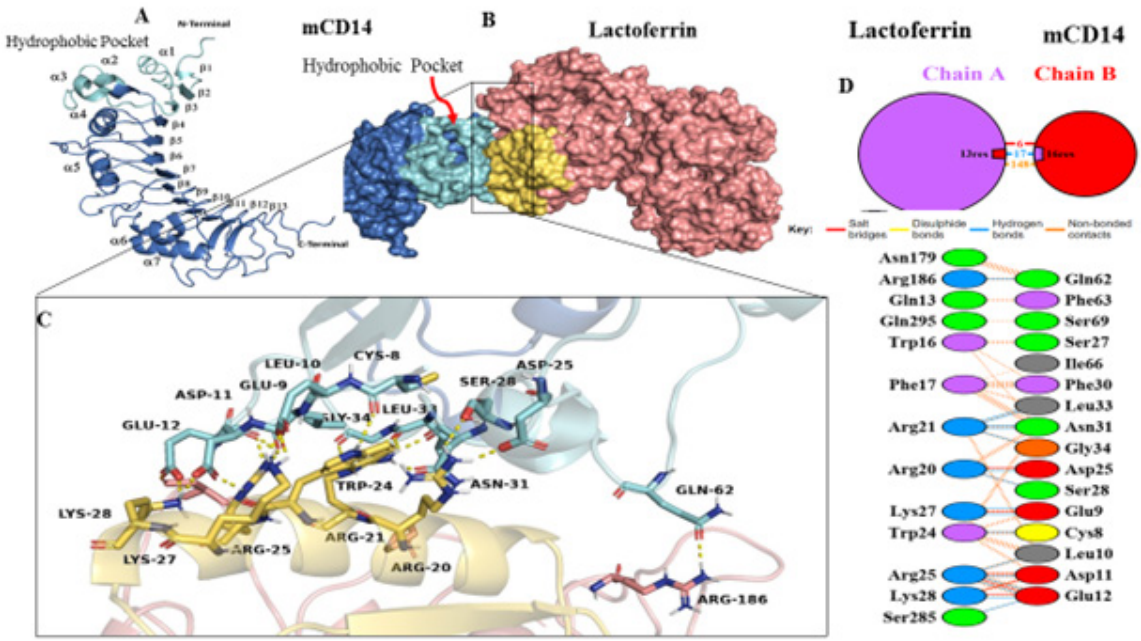

Figure 8. Lf and CD14 interaction. A, B) Tertiary structure of CD14 and Lf. C) Surface representation of the interaction between Lf and the CD14 receptor. Lf N-terminal binding region is shown in yellow color and CD14 receptor N-terminal region is shown in cyan color. D) The details of

Journal of Biochemistry (ISSN 2995-6536)

binding and important amino acids in the interaction between these two proteins are shown in linear representation. Docking was done using Cluspro website prepared by PyMol and PDBsum website.

Lf interactions to a selected gene promoter

The sequence of a part of the promoter of BAX, BAX, TP53, and SKP1 genes was obtained from the EPD website of the eukaryotic promoter database. First, four promoter sequences for different isoforms of the TP53 gene, one promoter sequence for the BAX gene, and two sequences for different isoforms of the BAK gene were used for the study, and finally, only one of the results was reported for the final analysis. The three-dimensional structures of the promoter sequences, which were done according to modeling based on the experimentally determined structure pattern, had good accuracy. Molecular Docking results using the PyDock web server between the promoter sequence and Lf protein are shown in **Table 2**. The numbers calculated by PyDock are a measure of the energy of interaction between Lf and the promoter regions, and the more negative the value of these numbers, the greater the binding tendency. Docking of Lf with isoform number 1 of the TP53-1 gene showed that Lf with the N-terminal region of amino acids 16-72 and also the region of amino acids 278, 284, and 285 interacted with bases 33 to 60 at the end of the promoter region of TP53 gene (Figure 9). The results of docking analysis showed that Lf from both the N-terminal and C-terminal regions interacted with bases 1 to 16 and 47 to 60 at both ends of the promoter region of the BAX gene (Figure 10). The results of docking analysis showed that Lf from amino acids 16 to 72 as well as amino acids 278, 284, and 285 interacted with bases 6 to 18 at the beginning of the BAK gene promoter region (Figure 11) .

Table 2. The numbers were obtained by the PyDock program, which are the scores of the best docking state between the promoter sequence and Lf protein.

Genes Name	TP53	BAX	BAK
PyDock Score (KJ/mol)	-233.396	-262.553	-219.105

Figure 9

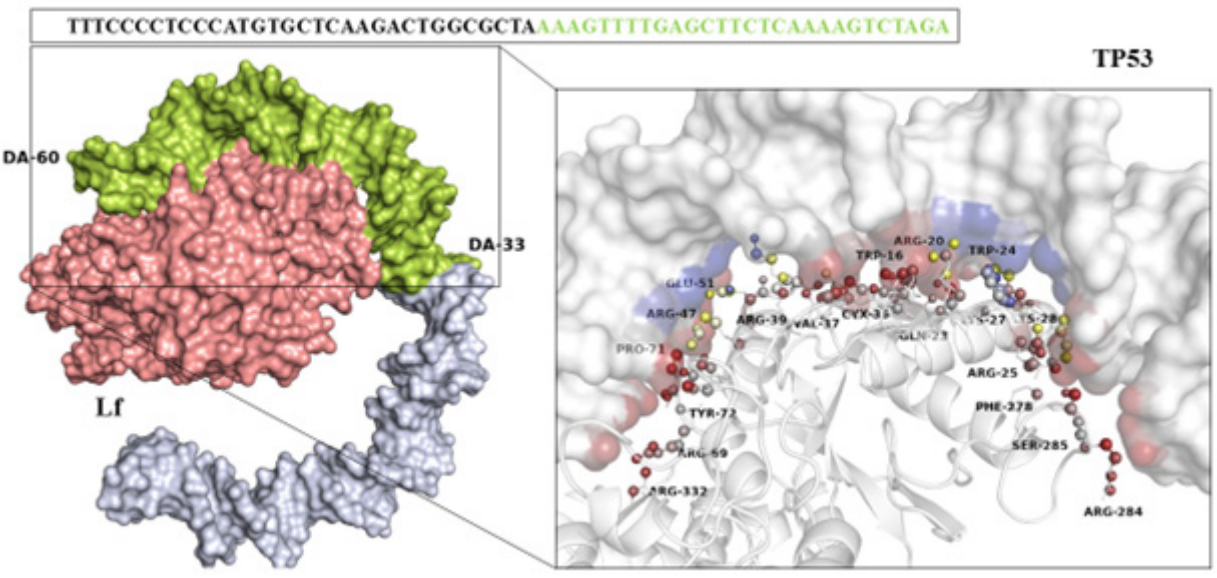

Figure 9. Surface representation of the interaction between Lf and the DNA of the promoter region of P53 gene isoform number one. The sequence of the interacting regions of the promoter is shown in green color and the important amino acids in the interaction are shown as a line and ball representation.

Journal of Biochemistry (ISSN 2995-6536)

Figure 10

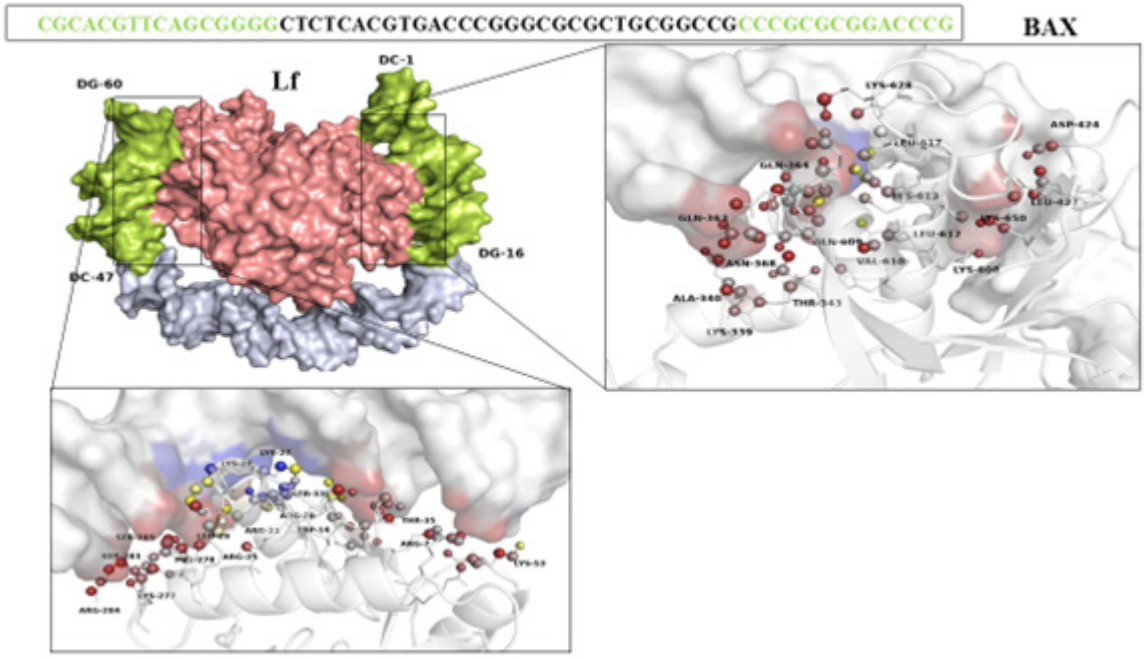

Figure 10. Surface representation of the interaction between Lf and the promoter region of the BAX gene. The sequence of the interacting areas of the promoter is shown in green colour and the important amino acids (N-terminal and C-terminal regions of Lf) in the interaction are shown in the form of linear and ball representations.

Figure 11

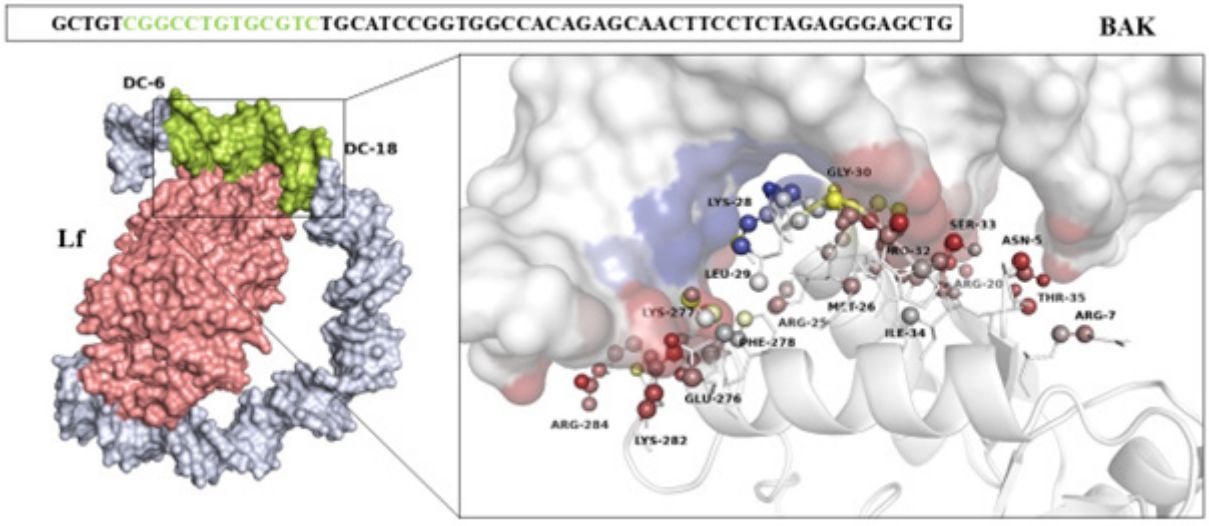

Figure 11. Surface representation of the interaction between Lf and the DNA of the promoter region of BAK gene isoform number one. The sequence of the interacting regions of the promoter is shown in green color and the important amino acids in the interaction are shown as line and ball representation.

Journal of Biochemistry (ISSN 2995-6536)

DISCUSSION

Nowadays, searching in existing databases and current computational methods is useful for developing new strategies for targeted treating diseases, including cancer and viral infection [37]. The study of interaction networks of proteins and genes by program applications is effective in drug design and provides researchers with a good insight into the mechanisms of drug action. The findings indicate that molecular and biochemical mechanisms related to cell death in cancer treatment should be determined before drug design [38]. Some important biological interactions occur in transient complexes, and hence experimental studies may be very difficult to determine, even when the structures of the protein components are known, so methods of docking studies have been developed [36].

Lf protein expression is also activated in some infections, including bacterial and viral infections. A region in the Lf gene promoter that is activated in response to infection has been determined. Lf gene expression is stimulated by bacterial membrane lipopolysaccharide and double-stranded RNA [12]. Studies suggest that Lf may be an effective treatment option for COVID-19 by binding to the spike protein of the virus and suppressing the ability of the virus to replicate [39]. In the present study, molecular docking was performed between the Lf protein and the specific cellular receptor of the COVID-19 virus, as well as between the spike protein of the virus and the cellular receptor. In the molecular docking study between Lf with the spike of the COVID-19 virus and the ACE2 receptor of human cells, the amino acids involved in the interaction between the spike and the ACE2 receptor were similar to the amino acids in the interaction between the spike and Lf. The binding between Lf and virus spike had the highest affinity, and also the binding strength of Lf with both spike and cell receptor was higher than the binding of spike with cell receptor. According to the results, the binding site of the spike to the receptor is the same as the binding site of Lf to the receptor, therefore Lf can be proposed to block the access of the spike for binding to the receptor. Since Lf can bind to the C-terminal region of the spike from its N-terminal domain, Lf can specifically block the region that binds to the cell receptor. Therefore, the N-terminal region of Lf can be an option to prevent spike binding to its receptor on the cell surface. Since the S2 subunit of nCoV-2019 is highly conserved and has 99% identity with the human SARS-CoV virus, and the S1 subunit shows about 70% identity, soluble ACE2 is a drug candidate that neutralizes the infection [40]. The amino acids located at the tip of the insertion section at the C-terminal domain of the S1 subunit of the spike are important in binding and have been preserved during evolution [41], and since Lf interacts with some of the amino acids in this section with a higher tendency, it can be considered as a suitable drug candidate to neutralize the viral infection. On the other hand, Lf with amino acids of the N-terminal region can connect with heparan sulfate and the carbohydrate part (glycoseaminoglycan) of the cell receptor which are the binding sites of the virus on the cell membrane, thus preventing the adhesion of the virus and its entry into the cell [3, 42]. The binding domain (RBD) present in the virus interacts with the peptidase domain (PD) of the ACE2 receptor through a polar interaction [43]. Piacentini et al stated that the inhibitory action of Lf is due to binding with ACE2 [44]. In addition, another point of attachment that facilitates virus attachment is the connection between viruses and heparan sulfate proteoglycan, which is exposed on the surface of the host cell, which is a primary connection that allows the accumulation of viral particles on the cell. Then the virus binds to the receptor and penetrates the cell [45]. The sites of virus attachment to cells are useful for understanding the mechanisms of action of Lf in the early stages of infection.

Lf can also prevent virus replication due to its ability to inhibit RNA polymerase, helicase, and viral 3CL proteases, which are very important for the normal cycle of virus replication. Also, Lf can increase the expression level of genes related to the cell's immune response against viral infection, thus stopping the infection [46]. Lf also plays a role in the regulation of the innate and acquired immune response due to the positive charge of the N-terminus, which can bind to the cell surface and affect the expression of pro- and anti-inflammatory cytokines [47].

Early studies in patients with severe COVID-19 disease suggest a reduction in the number and function of natural killer cells (NK), leading to reduced clearance of infected and activated cells and an uncontrolled increase in inflammatory markers that damage tissue [48]. It has been reported that Lf is capable of anti-viral and anti-tumor activity by increasing the amount of NK cells and other immune cells, including lymphocytes [49]. Increased activation of NK cells, CD4+, and CD8+ of T lymphocytes was observed after oral Lf treatment [50, 51]. Lf can positively regulate NF-κB, which is a transcription factor that regulates DNA transcription and cytokine production and also plays an important role in regulating the immune system [52]. In this way, by regulating NF-kB, it can prevent cytokine storms during the infection of the COVID-19 virus. Lf is also able to prevent cell apoptosis induced by the virus [53, 54]. Lf prevents the action of caspase 3 in the influenza A virus, which plays an important role in regulating apoptosis, thus Lf controls the apoptosis of infected cells [33]. Lf prevents the infection of herpes simplex viruses 1 and 2 (HSV1 and HSV2) by binding to heparan sulfate and glycosaminoglycan chondroitin sulfate on the cell membrane. Therefore, Lf acts in the initial stage of viral infection. Lf and lactoferricin have activity against HIV. Lf prevents the replication of the virus by preventing it from binding and entering the cell [33]. Lf

showed antiviral activity against enveloped RNA viruses such as SARS-COV [55]. Treatment with bovine Lf reduced virus load and virus-induced damage in the lungs and trachea [39]. Lf increased down-regulation signal pathways such as apical junction and TNF-α through the NF-κB/p53 pathway and corrected the disturbance in the expression level of inflammatory cells and adhesion-related genes [39]. Lf can be used as a food supplement which can reduce inflammation, balance the immune system, and restore the health of the microbiota barrier [56].

In this study, the structure of the cell receptor CD14 and the binding site of bacterial lipopolysaccharide, as well as how Lf binds to this receptor, were investigated. The results showed that Lf is connected with its N-terminus to the N-terminus region of the CD14 receptor, which contains lipopolysaccharide binding sites. This means that the binding site of bacterial lipopolysaccharide to the cell receptor is the same as the binding site of Lf on the cell receptor, so there can be a competition for binding between Lf and bacterial lipopolysaccharide. Lf with its N-terminus, which has positively charged amino acids such as arginine and lysine, attached to amino acids number 9 to 69 of the N-terminus of the CD14 receptor which includes lipopolysaccharide binding sites, and mostly includes negatively charged amino acids such as aspartic acid and glutamic acid. Therefore, with ion binding, which is a strong type of binding, it can compete with bacterial lipopolysaccharides for binding to the receptor. Lf has a direct antibacterial effect due to its direct interaction with the microorganism and especially due to its ability to bind to lipid A from LPS of the bacterial cell membrane, which increases its permeability into the cell [33]. In human and animal model studies, both *in vitro* and *in vivo*, the role of Lf in inhibiting host cell/LPS interactions has been proven. Lf has been shown to interact with the LBP domain, which mediates the binding of LPS to the CD14 receptor [28]. The active parts of Lf and lactoferricin B are those that have beta-sheet conformation in the presence of LPS. Thus, the β-sheet structure of Lf is more effective for initial contact with the bacterial membrane than the helical structure present in wild-type Lf [33].

In the present study, the results related to the docking of TP53, BAX, and BAK1 genes promoter and Lf protein showed that the level of Lf interaction with TP53 and BAX genes is higher with the binding tendency because PyDock scores which represent a measure of the interaction energy between Lf and promoter regions were more negative in these two bindings, which indicates a higher binding tendency. Lf interacted with the promoter of TP53, BAX, and BAK genes from the N-terminal region, which is the functional region of this protein. The result of molecular docking between the promoters of TP53, BAX, and BAK genes and Lf protein also showed that one or more amino acids present in the region 20-24 that are responsible for binding to DNA, interacted with the promoter region of this gene. The binding of Lf to DNA occurs under strict conditions with certain sequences of DNA specifically, and the interaction between Lf and specific sequences in DNA leads to the activation of gene transcription [5]. Therefore, according to the results of this type of docking and previous experimental studies, it can be said that Lf as a transcription factor increases the expression of the mentioned genes. Therefore, according to these in silico studies, it can be assumed that Lf can induce the expression of the wild type of the P53 gene or cause the reactivation of the mutant gene by acting on the P53 gene promoter, and also through its special activity to activate protein kinase by phosphorylating the p53 protein, it activates this protein and is somehow useful in the fight against cancer cells by inducing cell death. Inducing the reactivation of the mutated P53 gene in cancer is considered a useful strategy in the specific treatment of cancer. TP53 gene is a tumor suppressor gene that inhibits the growth of cancer cells through various mechanisms including induction of apoptosis and cell death. The p53 protein is a transcription factor known as the "guardian of the genome" due to its vital function in maintaining genomic integrity [57]. The TP53 gene is mutated in approximately half of human malignancies, including breast, colon, lung, liver, prostate, bladder, and skin. If the p53 protein is mutated, the cell cycle becomes unlimited and the damaged DNA is replicated, resulting in uncontrolled cell proliferation and cancerous tumors. p53 protein regulates the ability of cancer cells to proliferate, escape apoptosis, invade and metastasis [57]. Since p53 plays an essential role in regulating cell fate in response to DNA damage, therapeutic strategies that activate the p53-mediated pro-apoptotic pathway and/or suppress the dominant negative effect of mutant p53 on wild-type p53 are of interest. Phosphorylation of the p53 protein plays an important role in the regulation of apoptosis and cancer progression. Phosphorylation is induced in amino acid Ser46 in this protein, which is mediated by several kinases, including protein kinase (HIPK2), P38, and dual-specific tyrosine kinase regulatory kinase 2 (DYRK2) [57]. Lactoferrin increased the expression of p38 and JNK [2] therefore, lactoferrin can indirectly cause the phosphorylation of p53 protein and its activation. Lf can activate and positively regulate the P53 gene [58].

In previous studies, it has been determined that Lf interacts with two proteins, BAX and BAK [59], and Lf increased BAX and BAK gene expression in MCF7 breast cancer cells [60] which means that Lf has an effect on the mentioned proteins both at the gene level and at the protein level. Bax and Bak are the two main proteins of the apoptosis activation pathway. These two proteins control the permeability of the mitochondrial outer membrane through VDAC (voltage-dependent anion channel) and ANT (adenine nucleotide translocase) channels [61]. BAK and BAX genes are important genes in the mitochondrial-dependent apoptosis process. At the time of DNA damage,

Journal of Biochemistry (ISSN 2995-6536)

the p53 protein is phosphorylated as a transcription factor, which increases BAX gene expression and induces apoptosis [62].

Lf, having multiple biological activities, has always been a target for cancer treatment [63]. Lf prevents tumorigenesis through different mechanisms, including; regulation of the cell cycle, inhibition of angiogenesis, induction of apoptosis, regulation of the activity of natural killer cells (NK), and regulation of the immune system [22]. Many studies showed the presence of Lf receptors on the surface of tumor cells. Heparan sulfate of the cell membrane plays an important role in regulating the binding of Lf and lactoferricin with receptors [2]. One of the ways to treat cancer is to control or possibly end the unlimited and uncontrolled growth of cancer cells. Therefore, targeting apoptosis is the most successful non-surgical treatment, which is possible by stimulating pro-apoptotic molecules and inhibiting anti-apoptotic molecules [64]. It has been determined that the N-terminal region of about 40 basic amino acids is responsible for the role of apoptosis induction by Lf [17]. Lf can activate the nuclear factor NF-κB signalling cascade and as a result, it has caused the positive regulation of P53 gene expression in cervical cancer [65]. Lee et al. in 2009 showed that Lf can induce apoptosis by inducing the phosphorylation of JNK and P38 [58]. Lf has the activity of activating serine/threonine kinases, also Lf can phosphorylate kinases that are related to cell growth and survival [38]. Therefore, Lf, having the activity of activating serine/threonine kinases, can activate P38 kinase and the JNK kinase domain, both of which phosphorylate the p53 protein. In 2022, Rocha et al. showed that bovine Lf has an antitumor effect on prostate cancer cell line DU-145 [66]. Legrand et al reported that Lf activates intrinsic and extrinsic apoptotic pathways through the activation of various caspases. They showed that Lf induced apoptosis in colon cancer cells by activating caspase 8 in the extrinsic pathway [67]. In 2015, Amiri et al. reported that Lf was able to induce apoptosis in gastric cancer cell line AGS by 50 and 70% after 24 and 48 hours [68]. Also, Lf was able to induce apoptosis in KYSE-30 tracheal cancer cells [69]. Studies have shown that both the promoter regions of the Lf gene P1 for Lf and P2 for the second isoform of Lf (delta-Lf) were downregulated or silenced in several cancer cell lines. Cancer cells have a relatively high prevalence of genetic polymorphisms, gene mutations, and promoter hyper methylation in the Lf gene. The findings confirmed that there is a close relationship between Lf gene changes and increased incidence of cancer [2]. Therefore, the use of Lf as a new cancer marker may be useful in cancer diagnosis and also in assessing the prognosis of cancer patients at different stages. The amount of lactoferrin released from neutrophils can be detected in the stool sample. Lactoferrin in the stool sample can be used as a biomarker for inflammatory diseases as well as ulcerative colitis and Crohn's disease [32]. It has been found that lactoferrin levels increase significantly during chronic periodontitis, a chronic infectious inflammatory disease that eventually leads to the destruction of bone structures under the teeth, so measuring lactoferrin levels in saliva is It can be used as a biomarker to determine the amount of tooth bone destruction [70].

Lf reduces the level of cytotoxin and increases the antioxidant power of redox iron (FRAP) in the intracellular and extracellular space. In addition, Lf can fight the explosion of oxygen in neutrophils, which leads to the massive production of cell-damaging free radicals [71, 72]. Lf has a protective role against DNA damage directly and indirectly by affecting the human genome. These effects include interaction with DNA, iron homeostasis, cell cycle regulation, elimination of hydroxyl radicals, and antimicrobial and anti-inflammatory functions [73].

CONCLUSION

Lf with multiple functions can act against viruses and bacteria and also has anti-cancer activity. Lf can be absorbed both orally and by injection because its receptors are present in normal and cancer cells. Also, its functional region, which is related to the N-terminal and the active peptide of this part, lactoferricin, will remain in the human digestive system and can be active even after digestion with digestive enzymes. More importantly, it has immune compatibility with the host and does not provoke the immune system against itself. Therefore, as a suitable drug candidate, it can be used for both treatment and prevention as well as diagnosis of diseases resulting from viral, bacterial, and cancer infections. Its medicinal form can be taken as an oral supplement. For its greater stability and absorption of larger amounts and to prevent possible instability in the digestive system, its nano-encapsulated form can be used, and recent studies have confirmed its stability in this form. Nano-encapsulated Lf retains all its medicinal properties, so it can be used as a therapeutic agent in combination because it has no side effects.

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Declarations

Ethical statement

Not applicable.

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Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Fatemeh Moradian and Havva Mehralitabar. The first draft of the manuscript was written by Fatemeh Moradian and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Competing Interests

Authors declare they have no financial interests.

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