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Original Article

Sequence Analysis of Hot Spot Regions of Spike and RNA-dependent-RNA polymerase (RdRp) Genes of SARS-CoV-2 in Kerman, Iran

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Abstract. ***Background:*** **Mutations in the SARS-CoV-2 genome might influence pathogenicity, transmission rate, and evasion of the host immune system. Therefore, the purpose of the present study was to investigate the genetic alteration as well as assess their effects on the receptor binding domain (RBD) of the spike and the putative RNA binding site of the RdRp genes of SARS-CoV-2 using bioinformatics tools.**
Materials and Method: **In this cross-sectional study, 45 confirmed COVID-19 patients using qRT-PCR were included and divided into mild, severe, and critical groups based on the severity of the disease. RNA was extracted from nasopharyngeal swab samples using a commercial kit. RT-PCR was performed to amplify the target sequences of the spike and RdRp genes and sequence them by the Sanger method. Clustal OMEGA, MEGA 11 software, I-mutant tools, SWISS-MODEL, and HDOCK web servers were used for bioinformatics analyses.**
Results: **The mean age of the patients was 50.68±2.73. The results showed that four of six mutations (L452R, T478K, N501Y, and D614G) in RBD and three of eight in the putative RNA binding site (P314L, E1084D, V1883T) were missense. In the putative RNA binding site, another deletion was discovered. Among missense mutations, N501Y and V1883T were responsible for increasing structural stability, while others were responsible for decreasing it. The various homology models designed showed that these homologies were like the Wuhan model. The molecular docking analysis revealed that the T478K mutation in RBD had the highest binding affinity. In addition, 35 RBD samples (89.7%) and 33 putative RNA binding site samples (84.6%) were similar to the Delta variant.**
Conclusion: **Our results indicated that double mutations (T478K and N501Y) in the S protein might increase the binding affinity of SARS-CoV-2 to human ACE2 compared to the wild-type (WT) strain. Moreover, variations in the spike and RdRp genes might influence the stability of encoded proteins.**

Keywords: COVID19; SARS CoV-2; RdRp; Spike; Iran; putative RNA binding size; receptor binding domain; RBD.

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Introduction. The SARS-CoV-2 outbreak occurred in Wuhan, China, in December 2019 and quickly became a pandemic by spreading to all countries worldwide, resulting in significant public health problems and even economic distress for nations.[1,2] As of the end of the year, more than 608 million confirmed COVID-19 cases were reported worldwide, with 6.5 million confirmed deaths.[3]

Coronaviruses are divided into three genera, including Alpha, Beta, and Gamma coronaviruses; SARS, MERS, and SARS-CoV-2 are members of the Beta coronavirus. All coronaviruses have a genome consisting of a positive-sense single-strand RNA molecule. SARS-CoV2 encoded four structural proteins, including spike, 16 nonstructural proteins (NSP), and nine accessory proteins, including the RNA-dependent RNA polymerase (RdRp) (also named nsp12).[4]

The spike glycoprotein mediates receptor binding and fusion of the viral and cellular membranes.[4] In addition, it is subject to proteolytic cleavage by host proteases (i.e., trypsin and furin) at two sites at the polybasic furin cleavage site (PCS), located at the boundary between the S1 and S2 subunits (S1/S2 site). The binding of ACE2 with Spike S1 (Amino Acids (AA): 15–685) protein allows the virus to adhere to the lung epithelial cells. A small, independently folded subdomain of S1, described as the receptor-binding domain (RBD), directly binds ACE2 (AA: 320–540) when the virus engages the target cells.[5] The virus is predicted to be zoonotic in origin, and mutations in the surface glycoprotein structure enable its transmission to human hosts.[6]

There has been evidence of SARS-CoV-2 mutations that have a substantial functional effect on the virus.[7] A mutation resulting in an AA substitution in the spike protein (D614G) emerged early in the epidemic and spread rapidly through Europe and North America, particularly.[8] During a joint study in the United States and the Netherlands in September 2020, researchers discovered that the D614G mutation of the spike protein of SARS-CoV-2 was predominant and affected disease presentation.[9] Two important mutations in the RBD at positions R407I and A930V have been identified in India, increasing the molecule's dependence on its receptor.[10] Another mutation in the RBD position was N501Y, which American researchers showed to neutralize human convalescent or post-vaccination sera in vitro.[11,12] The emergence and spread of a SARS-CoV-2 lineage that contains several nonsynonymous spike mutations, including mutations that affect key sites in the RBD (resulting in K417N, E484K, and N501Y substitutions) in South Africa may have functional importance.[4] Specifically, residues G431 and S514 in SARS-CoV-2 RBD are important for S protein stability. An experimental study indicated that an S missense mutation such as D614G contributed to the dominant pandemic form and could stabilize the entire S protein.[13]

SARS-CoV-2 RdRp is the prime constituent of the replication/transcription machinery prone to mutation.[14] RdRp is a primary target for antiviral inhibitors like Remdesivir,[15] Favipiravir, Galidesivir, and Ribavirin,[16] as well as some other potential drugs such as Filibuvir, Cepharanthine, Simeprevir, and Tegobuvir.[16] The RdRp mutation may alter the rigidity of the RdRp protein structure, which can exert its effects through altered interaction with the RNA pattern or with other components of the transcription/replication machine, thereby altering the mutation rate.[17] A comparative analysis shows that some mutations are specific to a certain region; for example, T265I, P5828L, and Y5865C are unique to the United States and have not been reported from Europe or Asia, while P4715L is predominant in Europe. Moreover, four significant mutations T265I (nsp 2), P4715L (nsp 12), P5828L, and Y5865C (both at nsp 13), were identified in important nonstructural proteins, which function either as replicas or helicase.[18]

As the virus spreads to new places, it alters its protein sequence by introducing mutations in its genome that help it survive better in the host.[19] As an efficient unique pathogen, this virus often mutates its proteins so that it can still infect the host cells, change its pathogenicity and transmission rate, and evade the host immune system.[20] Even when profitable strategies are discovered and engaged, viruses' high rate of genetic change frequently leads to drug resistance or vaccine escape.[21] With cautious optimism at the recent decline in cases, there is still anxiety about subsequent waves of infections after the relaxation of containment procedures in various cities and countries.[22]

Although the mutation rate in SARS-CoV-2 is much lower than that of other RNA viruses, including seasonal flu viruses, knowing these mutations can help us create targeted treatments through more effective vaccines and antiviral therapy and reveal its pathogenesis, transmission rate, and spread. Therefore, our studies focused on the mutation rate and assessed their effects on the spike's receptor binding domain (RBD) and the putative RNA binding site of the RdRp genes of SARS-CoV-2 using bioinformatics tools.

Materials and Methods.

Study population. From July 28 to September 1, 2021, 45 COVID-19 patients were recruited consecutively from

Afzalipour Hospital, affiliated with Kerman University of Medical Science. The selected patients were divided into three groups, mild, severe, and critical, based on the disease severity according to the World Health Organization (WHO) criteria.[24] The inclusion criteria were as follows: 1) patients that met the diagnostic standard for SARS CoV-2 virus based on the Center for Disease Control and Prevention (CDC) definition as an acute respiratory disease with laboratory-confirmed SARS CoV-2 infection by qRT-PCR;[25] 2) positive results from throat swabs; 3) patients with an intense cough with bloody or purulent sputum, chest pain, severe vomiting, diarrhea, dehydration, and dyspnea; 4) patients with complete clinical information; 5) patients who did not complain about other infectious diseases, history of hematological diseases, or immune system disorders; and 6) patients who were inoculated with any of the Corona vaccines. A structured questionnaire was used to collect demographic characteristics, temperature, number of breaths per minute, weekly exercise, smoking, and a history of underlying disease. Written informed consent was obtained from each participant.

Sampling. Nasopharyngeal samples were taken from all patients using a Dacron swab and then transferred to Viral Transporter Media (VTM) in a cool box at the research laboratory in the School of Medicine, Kerman University of Medical Sciences.

RNA extraction. Viral RNA was extracted from throat samples (one sample, one patient) using a highly pure Viral RNA Kit (Roche, Product no: 11858882001) based on manufacturer instructions.

Primer design and reverse transcriptase polymerase chain reaction (RT-PCR). The RBD domain sequence, the spike-glycoprotein gene-cleavage site, and the putative RNA binding site of RdRp of the ORF1ab gene were extracted from the NCBI database. The AlleleID program also designed primers for the desired domains.

The region of the RBD-cleavage site of the S glycoprotein and the putative RNA binding domain of the RdRp was amplified by PCR methods. The primer sequences, annealing temperatures, and product size are shown in **Table 1**.

This reaction was performed in two steps. In the first step, the reverse transcriptase (RT) PCR method was performed in one step, and the components were combined in a single tube. The RT-PCR was performed in a standard protocol in a 25 µl volume containing the primer, RNA template, and 2X one-step RT-PCR master mix (Qiagen, Germany). In the second step, the conventional PCR technique with the specific internal primers on previous products and Taq DNA Polymerase Master Mix RED 2X (Ampliqon) was performed. 12.5 µl of a 2×master mix, 10 pmol of each primer, and 150 ng of extracted RNA were used. The reaction was performed in an Eppendorf thermal cycler under the following conditions: First denaturation for 5 min at 95°C followed by 40 cycles: 40 s at 95°C, 35 s at Ta °C, and 40 s at 72°C. A final extension at 72°C for 5 min was performed at the end of the cycles.

The PCR products were electrophoresed on a 1.5% agarose gel. A 100-bp molecular weight marker was used for band detection. The target bands were sliced for gel purification, which was performed by the MinElute PCR Purification Kit (Qiagen GmbH) according to the manufacturer's protocol.

Sanger Sequencing. Purified amplified products were sent to the 1st BASE Company (Gemini) for Sanger sequencing. Of 45 samples, 39 results for the RBD–cleavage site and the putative RNA binding site were clear and perfect and used for sequence analysis.

Mutational analysis. The sequences obtained from the studied region were aligned and complemented with CLC 6 and Clustal OMEGA. To confirm the results, we compared the sequences with the existing database using the online BLAST search tool (www.ncbi.nlm.nih.gov). To find the related mutations of the RBD–cleavage site and putative RNA binding site, we compared the sequences with the reference strain Wuhan-Hu sequence using the Clustal OMEGA algorithm.

To identify the nucleotide variations, we performed multiple sequence alignments using Clustal OMEGA,[26] and the sequence of the strain Whuhan-Hu-1 (GenBank accession number: 045512) was used as a reference genome. The alignment file was analyzed using the MVIEW program of Clustal OMEGA.[27]

Detection of mutation Spectrum. Clustal OMEGA was

Table 1. The sequences of the primers used for amplification of RBD–cleavage site of the spike and Putative RNA binding site of the RdRP using the PCR method.

Primer	Sequence (5′ -3′)	Ta (°C)	Position at the whole genome	Product size
Putative RNA binding site	F: GTGTTCTCTTATGTTGGTT	58.1	1228-2310	1083
	R: AGTAGGTTGTTCTAATGGT			
RBD-cleavage site	F: TCTTCTTCAGGTTGGACAGC	58.6	760-1810	1050
	R: ATCACGGACAGCATCAGT			

used again for the multiple sequence alignment of each protein, which was further analyzed by MVIEW. The amino acid variations were identified in each protein by comparing it to the protein of the reference strain. Further, both nucleotide and amino acid variations were compared to study the types of mutations.

Prediction of mutational effects. The structural and functional effects of the missense variants and the stability change were analyzed using different prediction tools. I-mutant was employed to analyze the stability change with all the parameters set to default.[28] Additionally, Mutpred2 was adopted to predict the molecular consequences and functional effects of these mutations.[29]

Homology modeling of spike proteins and model validation. The BLASTp program at the NCBI interface (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) was used to find the most suitable template for homology modeling. Getting the protein databank reservoir (PDB), we identified spike protein with PDB ID: 6M0J as a suitable template, as it has 99.59% sequence similarity and 94% coverage with the target sequence. The homology modeling of all mutant spike proteins, along with the spike protein of the reference, was done using SWISS-MODEL.[30] The predicted model was validated by adopting Rampage and ERRAT.[31]

Molecular docking of Spike Protein with ACE2 receptor. The molecular docking approach was employed to investigate the interaction of mutant spike protein with the human ACE2 receptor. First, the crystal structure of human ACE2 (PDB ID: 6D0G) was obtained from the Protein Data Bank (PDB), and PyMOL was used to clean the structure to remove all the complex molecules and water.[32] The HDOCK webserver was used to predict the interaction between Spike protein and the human ACE2 receptor through protein-protein molecular docking.[33]

Phylogenic analysis. Phylogenetic and sequence analysis based on RBDs and RdRp was performed from all 39 clear and perfect isolates. In the phylogenic analysis, we considered 6 variants of concern (VOCs), including B.1.1.529 (UZI26581.1), P.1 (QXU68443.1), B.1.617.2 (UAL04647.1), B.1.1.7 (UFQ05186.1), B.1.351 (UFQ05198.1), and whuhan strain (YP 009724390.1) as reference sequences. All sequences were clustered using MEGA 11 software. Finally, the evolutionary history was inferred using the Maximum Likelihood method as a statically model, the bootstrap method as a type of phylogenic (~1000 bootstrap), and the Tamura-Nei model as a substitution method.

Result.

Demographic characteristics of the subjects. In this cross-sectional study, 45 COVID-19 patients were included. Of these, 39 samples had clear perfect sequence results with an average age of 50.38 ± 2.13, including 21 males and 18 females (**Table 2**).

Mutation Spectrum of SARS-CoV-2 isolates. Of 45 PCR products sent for sequencing, 39 samples had clear perfect sequencing results and were used for analysis. Analysis of all 39 Kerman isolates revealed a total of 8 single nucleotide variants in the putative RNA binding site and 6 in the RB-cleavage site. Besides, one deletion was also found in those isolates, which was responsible for the deletion of putative RNA binding site isolates (**Table 3**). Three of the eight putative RNA binding site mutations were missense mutations, and five were synonymous. In addition, four of the six mutations in the RBD domain were missense, and the other two were synonymous (**Table 3**).

Mutational effects. Mutational effects analysis of the three missense mutations located in the putative RNA binding site revealed that two of them were responsible for decreasing structural stability and another for increasing it. Furthermore, four missense mutations occurred in the RBD and cleavage site domains; three of them were responsible for decreased structural stability, and one for increased structural stability (**Table 4**).

Prediction and validation of the homology models. Five models were generated using the template PDB ID: 6M0J: one for the spike protein of the reference strain and the other four for three different mutant isolates from Kerman. Mutant models 1, 2, 3, and 4 are designed for L452R, T478K, N501Y, and D614G mutations, respectively. The validation assessment scores of these four models were mostly similar to the template, which

Table 2. Demographical characteristics of the three study groups and healthy volunteer control group.

Group		Mean Age	SEM	IQR	p-value
Control		48.6	1.3	24.1	0.4
Total patients		50.7	1.6	23.6	
Patients	Mild	49.8	2.1	21.7	0.7
	Severe	51.4	2.7	25.3	
	Critical	50.2	2.5	18.4	

Table 3. All mutations found in ORF1a and RBD domain-cleavage site of the 39 isolates compared to the reference strain.

No	Mutation	Protein	Amino acid change	Mutation type
1.	14409:T > C	ORF1b	P314L	Missense
2.	15325:C > T	ORF1b	No change	Synonymous
3.	15739:C > T	ORF1b	No change	Synonymous
4.	15896:C > T	ORF1b	No change	Synonymous
5.	17020: G > T	ORF1b	E1084D	Missense
6.	18878:C > T	ORF1b	No change	Synonymous
7.	19405: G > A	ORF1b	V1883T	Missense
8.	22445:C > T	ORF1b	No change	Synonymous
9.	1103-1128: DEL	ORF1b	Whole protein deletion	Deletion
10.	22469: G > T	RBD	No change	Synonymous
11.	22917: T > G	RBD	L452R	Missense
12.	22995: A>C	RBD	T478K	Missense
13.	23063 A > T	RBD	N501Y	Missense
14.	23321:C > T	RBD	No change	Synonymous
15.	23403:A > G	Cleavage site	D614G	Missense

Table 4. Prediction of the mutational effects on the structural stability.

protein	Amino acid changes	SVM2 Prediction Effect	DDG Value (kcal/mol)
ORF1b Polyprotein	P314L	Decrease	-0.81
ORF1b Polyprotein	E1084D	Decrease	-0.71
ORF1b Polyprotein	V1883T	Increase	-0.23
RBD	L452R	Decrease	-0.45
RBD	T478K	Decrease	-0.53
RBD	N501Y	Increase	-0.19
Cleavage site	D614G	Decrease	-0.63

Table 5. Model validation assessment score.

Structures	Rampage Score		ERRATA Score
	Favored Region	Allowed Region	
Template	96.1%	3.8%	80.5%
Wild type	92.7%	5.8%	82.4%
Mutant model 1	93.4%	4.6%	79.4%
Mutant model 2	91.7%	5.9%	88.9%
Mutant model 3	92.3%	5.6%	84.9%
Mutant model 4	90.9%	6.9%	86.3%

Table 6. Molecular docking results of the human ACE2 receptor against wild-type and mutant spike proteins of SARS-CoV-2.

models	variations	HDOCK Score
Model 1	Wild type	-136.9± 2.5
Model 2	L452R	-139.9± 7.3
Model 3	T478K	-143.1 ± 1.5
Model 3	N501Y	-142.3 ± 0.9

proved the reliability of these models (**Table 5**).

Analysis of the interaction between spike proteins and human ACE2 receptor. The HDOCK server was used to predict the interaction between the 3D models of reference spike proteins ,mutant models, and the human ACE2 receptor. This molecular docking analysis confirmed that the T478K mutation had the highest binding affinity (-143.1), followed by N501Y (-142.3) and L452R (139.9). For three spike protein models, this

study showed that the spike protein domain, rather than the complete protein, amino acids 345 to 527, was involved in the interactions (**Table 6**).

Phylogenetic analysis. The RBD and putative RNA binding site sequencing results of the current study were compared to the world's registered prevalent concern variants, and detailed specifications are shown in **Figures 1** and **2**. According to the comparison results and different variants, the sequences confirmed the introduction of species by overcoming Delta species. It was found that most SARS-CoV-2 viruses circulating in Kerman, Iran, from July to September 2021 were similar to the Delta variant (B.1.617.2), with 90% and 98% genetic homology, respectively (**Figures 1** and **2**).

The prevalence of RBD VOCs with Pangolin Lineages B.1.617.2/Delta was 89.74%, and B.1.1.529/Omicron (5.10%) (**Figure 1**). In two samples (5.1%), in addition to the Delta variant, some RBD mutations of the Omicron variant (N501Y and T478K) were added to this type in patients in this area that might gradually dominate the previous species. Also, in a similar pattern, the prevalence of putative RNA binding sites of VOCs was 81.39% for B.1.617.2/Delta (**Figure 2**).

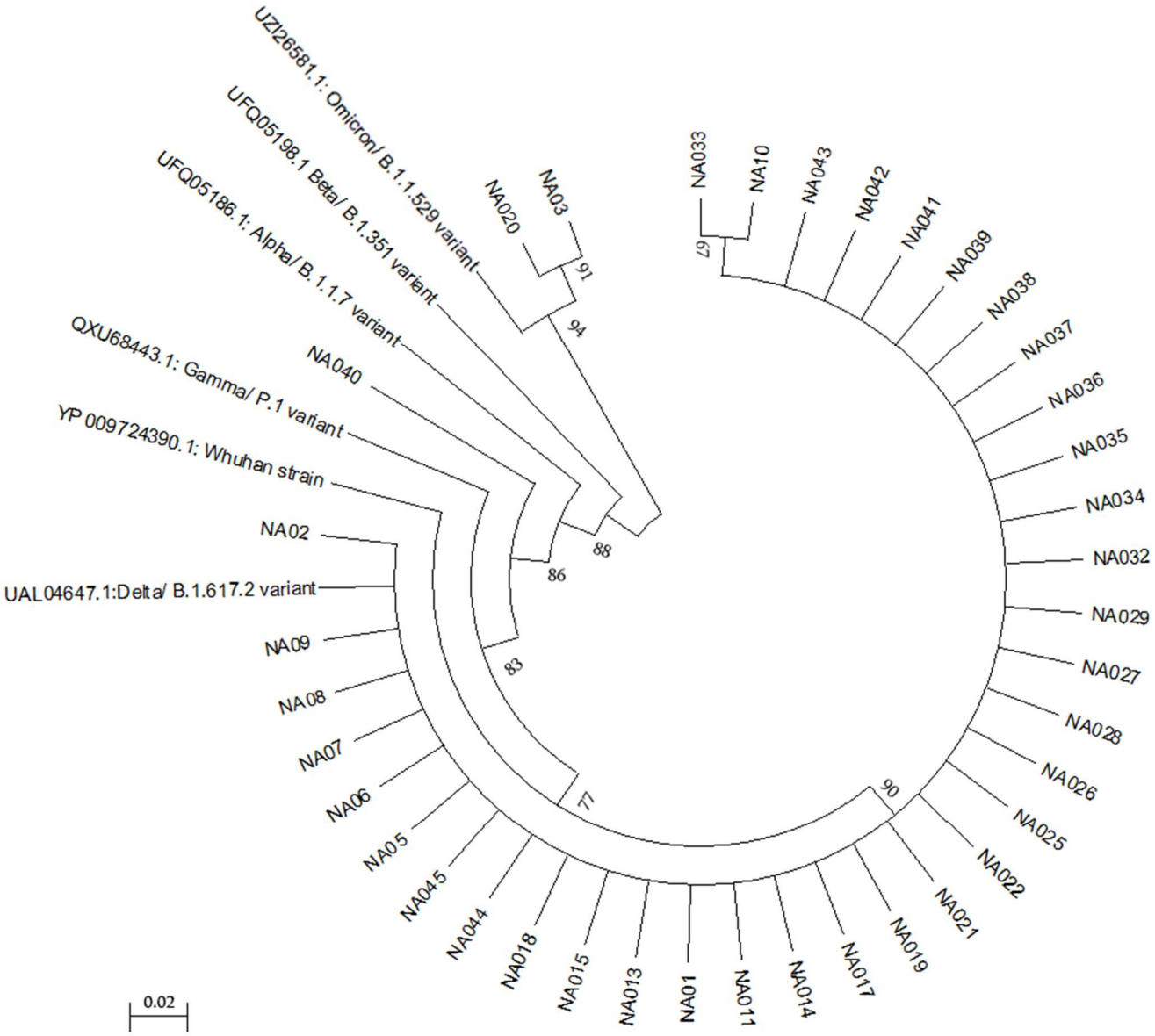

Figure 1. RBD phylogenetic tree. Evolutionary relationships among SARS-CoV-2 RBD, Kerman, Iran, 2021. The phylogenetic tree was generated using MEGA software v11, the Maximum Likelihood method (~1000 bootstrap), and the Tamura-Nei model. The phylogenetic tree was generated using MEGA software v11, the Maximum Likelihood method (~1000 bootstrap), and the Tamura-Nei model, which yielded the same results as shown in Figure 2.

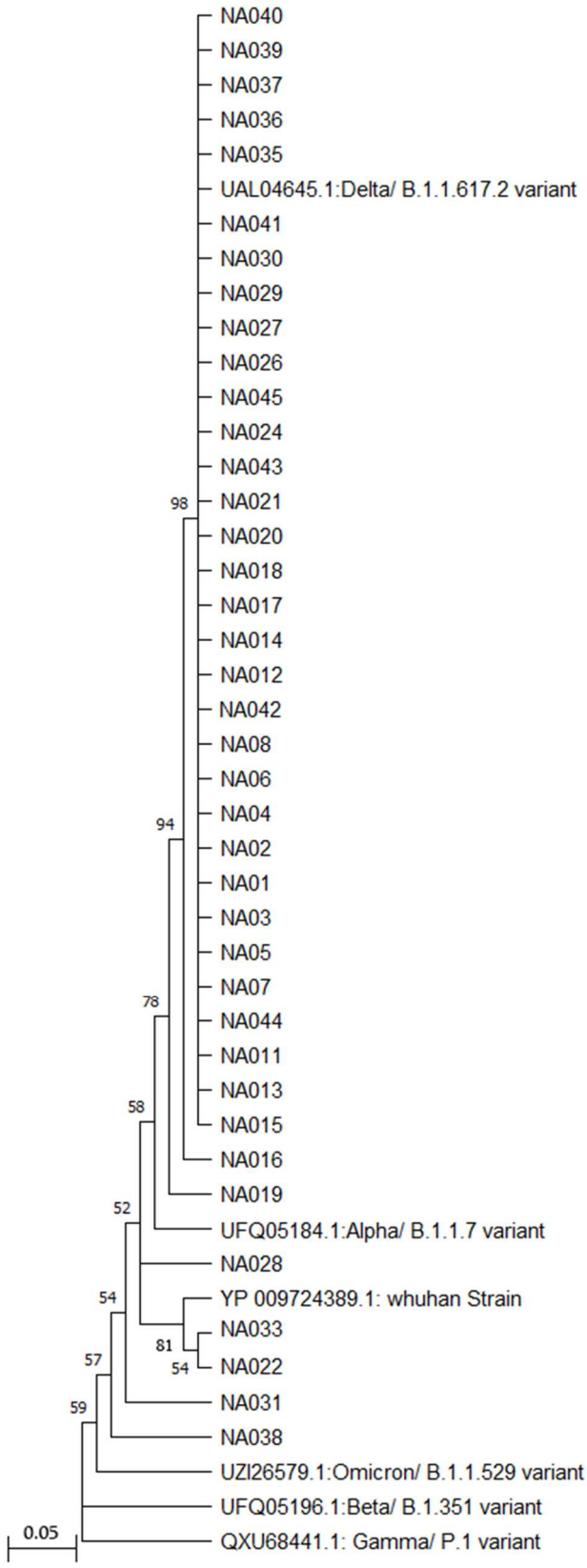

Figure 2. Putative RNA binding site phylogenetic tree. Evolutionary relationships among SARS-CoV-2 RBD, Kerman, Iran, 2021.

Discussion. SARS-CoV-2 has become a global challenge, affecting millions and taking thousands of lives daily. Along with other studies, viral and host genetic studies can give a significant clue to understanding the pathogenesis of COVID-19 and combating SARS-CoV-2.[34–36] In addition to the critical therapeutic target, the genomic sequence data may provide insights into the pattern of global spread, dynamics of evolutions, transmission rate, escape of the host defense, and importance in unraveling the molecular pathogenesis mechanism of SARS-CoV2.[37]

In this study, we found some mutations in the SARS-CoV-2 isolated in Kerman, Iran, which may give insights into the transmission of SARS-CoV-2, the genetic diversity of the S gene (RBD and cleavage site domain), and ORF1ab (putative RNA binding) and predict the impacts of mutations. A total of 15 mutations were identified with a deletion, of which less than half were synonymous (7 mutations). Among the six mutations found in the RBD domain and cleavage site of the S glycoprotein, L452R, T478K, N501Y, and D614G were identified as missense mutations. This variant, which has evolved to become the predominant genotype worldwide, can evade cellular immunity, have high infectivity, and increase host glycolysis and fusogenicity, increasing the number of hospitalized patients with high morbidity and mortality.[38,39] These mutations also had the potential to evade the protective antibodies derived from the convalescent sera or vaccine origin, as well as those used in therapy, which could favor virus expansion and compromise infection control.[40] The results of a study have revealed a set of mutations, such as D614G and P314L, that influence the outcomes of SARS-CoV-2 infection and guide the development of SARS-CoV-2 vaccines.[41]

Moreover, our molecular docking analysis revealed that these mutations in the RBD domain affected the interaction with the ACE2 receptor, suggesting that this mutation is more responsive to hACE2, indicating the potential for transmission. T478K (-143.1) has the highest binding affinity, followed by N501Y (-142.3) and L452R (-139.9). In this regard, a study indicated that some mutations such as Q493R, N501Y, S371L, S373P, S375F, Q498R, and T478K significantly contributed to high binding affinity with human ACE2, which is consistent with our results.[42] Another study showed that mutations T478K and N501Y, particularly, had the highest binding affinity to Glucose Regulated Protein 78 (GRP-78) as a co-receptor for virus attachment to the host cell.[39]

Large deletion mutations can cause a defect in the production and function of the desired protein; some scientists have suggested that large deletions in OFR1ab may not encode the target protein.[43] Large deletion mutations have also been found in other scientists' studies; for instance, an 80-nucleotide deletion in ORF7a

was also reported in a study conducted in Arizona.[44] Previous studies have shown that the lack of these side proteins (ORF8b) has adverse effects on viral replication, pathogenesis, and structural expression of the protein.[45] In another study, researchers indicated that loss of some of the SARS-CoV-2 proteins, such as ORF1a and ORF7, could cause a much more significant restriction of the spread of the virus into the host and may also lead to less pathogenicity of the virus, resulting in a meager infection rate and mortality compared to the other countries, which are consistent with the study conducted by Keng and his colleagues.[45]

Furthermore, compared to the reference, variations in the putative RNA binding site of the RdRp caused substitutions of one or more amino acids in the isolates from Kerman, Iran. Among the eight mutations found in the Putative RNA binding site, mutations P314L, E1084D, and V1883T were identified as missense ones. Some of the mutations were found to affect the structural stability of the proteins rather than alter their molecular functions, and some of those altered their molecular functions. The putative RNA binding site in ORF1ab is essential for replicating the viral genome into a helical ribonucleocapsid (RNP).[46] These functions may not be affected much by those mutations; Mutpred2 predicted that these mutations did not alter any molecular consequences of the proteins, which is consistent with the study conducted by Wrapp.[47] Parvez and colleagues' molecular docking analysis revealed no significant interactions between the putative RNA binding site and the genome.[48] Doga Eskier et al. reported that the third most common RdRp, 15324C>T, decreased the mutation rate created by RdRp, while the 14408C>T mutation had the opposite effect. They also suggested that the 14408C>T mutation contributed to co-mutations' dominance in Europe and elsewhere.[49]

The S gene displays higher tolerance for positive selection in mutant isolates early during the appearance of the double mutant genotype. It undergoes increasing negative selection over time, whereas the RdRp region in the mutant isolates shows strong negative selection throughout the pandemic.[50]

Conclusions. Our findings suggest that mutations may reduce the stability of spike and RdRp, whereas variations such as N501Y and V1883T may increase stability. Moreover, according to various homology models, these homologies were similar to those in the WT strain. Furthermore, the mutated structures (T478K and L452R) that were seen in the RBD domain might increase the binding affinity of SARS-CoV-2 to human ACE2 compared to WT[51] because of the significant changes in the electrostatic and van der Waals (vdW) interactions. Further studies are recommended to clarify the results.

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Ethics statement. The study was approved by the local Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1400.737).

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