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Original Article

Novel Mutations in the Non-Structure Protein 2 of SARS-CoV-2

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Abstract. ***Introduction*: Mutation in the genome of SARS-CoV-2 may play a role in immune evasion, pathogenicity, and speed of its transmission. Our investigation aimed to evaluate the mutations that exist in the NSP2.**
***Materials and Method*: RNA was extracted from nasopharyngeal swabs from 100 COVID-19 patients. RT-PCR was performed on all samples using NSP2-specific primers. Following gel electrophoresis, the bands were cut, purified, and sequenced using the Sanger method. After sequencing, 90 sequences could be used for further analysis. Bioinformatics analysis was conducted to investigate the effect of mutations on protein structure, stability, prediction of homology models, and phylogeny tree.**
***Results*: The patients' mean age was 51.08. The results revealed that 8 of the 17 NSP2 mutations (R207C, T224I, G262V, T265I, K337D, N348S, G392D, and I431M) were missense. One deletion was also found in NSP2. Among NSP2 missense mutations studied, K337D and G392D increased structural stability while the others decreased it. The homology-designed models demonstrated that the homologies were comparable to the sequences of the Wuhan-HU-1 virus.**
***Conclusion*: Our study suggested that the mutations K337D and G392D modulate the stability of NSP2, and tracking viral evolution should be implemented and vaccine development updated.**

Keywords: SARS-CoV-2, Non-structure protein 2, NSP2, Wuhan-HU-1.

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Introduction. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of the COVID-19 disease, is a highly contagious respiratory pathogen.[1] COVID-19 initially emerged in Wuhan, China, in December 2019 and has spread rapidly worldwide, leading to a global pandemic.[2,3] COVID-19 symptoms can manifest in a range of mild to severe forms, including fever, cough, shortness of breath,

fatigue, loss of taste or smell, and more rarely, gastrointestinal bleeding, pneumonia, acute respiratory distress syndrome (ARDS), and even death.[4–6] As of May 21, 2023, approximately 766 million cases of SARS-CoV-2 have been recorded, along with 6.9 million reported deaths.[7,8] The genome sequencing analysis of SARS-CoV-2 and SARS, conducted through the novel Coloured Genomic Bootstrap (CGB) barcoding method, reveals that despite the presence of genomic regions with mixed ancestry derived from horseshoe bat viruses, a predominant over 97% of their genomes originates from bats located in Yunnan, China.[9,10] However, another possibility is that the virus could have infected other mammals and transferred to the human population through the live animal market in Wuhan.[11,12]

The SARS-CoV-2 genome has an approximate size of 29.8 kb and consists of 14 open reading frames (ORFs).[13] ORF1a and ORF1b are cleaved into 15 non-structural proteins (NSP) including NSP1 to NSP10, and NSP12 to NSP16, by enzymatic function of NSP3 (papain-like protease) and NSP5 (chymotrypsin like protease).[14,15] The NSP2 disrupts host signaling during infection by interacting with Prohibitin 1 (PHB1) and Prohibitin 2 (PHB2), which are components of the mitochondrial prohibitin complex.[16,17] In addition, NSP2 plays a role in regulating calcium homeostasis within cells, post-transcriptional suppression, and associates with seven cellular proteins involved in vesicular trafficking.[14,18,19] Finally, NSP2, by initiating translational suppression, not only enables SARS-CoV-2 to evade the Interferon type I response but also contributes to inflammation by activating NF-κB.[20,21]

SARS-CoV-2 mutations can significantly affect viral circulation, immune evasion, and pathogenicity.[22] Frequent genetic variations in viruses can often result in drug resistance or the evasion of effective vaccination strategies.[23–25] As the virus spreads, it can help to survive in the host cell by introducing mutations and altering the structure of its proteins.[26] Viral polymerase, due to its limited or absent proof-reading activity, can lead to frequent mutations.[27] One of the key areas of concern has been the genetic variability in SARS-CoV-2 genomes. Recent research has identified sixty-one mutations, primarily concentrated in NSP3, RNA-directed RNA polymerase (RdRp), and Nucleocapsid proteins.[28] In-depth analysis of 59,541 SARS-CoV-2 genomic sequences revealed significant mutations, with certain mutations such as T85I and Q57H proving deleterious, while P323L exhibited a stabilizing effect, offering insights into the virus's evolutionary dynamics and potential impact on pathogenesis.[29] The rapid mutation rate of SARS-CoV-2 has resulted in the emergence of new viral variants, particularly in the spike protein's receptor-binding domain (RBD). These mutations can potentially enhance viral transmission, increase disease severity, and potentially reduce the effectiveness of immune responses, monoclonal antibody treatments, and vaccines.[30] Additionally, distinctive mutations in the SARS-CoV-2 ORF1ab polyprotein (265 T→I, 4715 P→L, 5828 P→L, and 5865Y→C) serve as a signature for the United States, altering nonstructural protein structures and emphasizing their relevance in antiviral therapeutic design.[31]

The absence of the NSP2 protein may compromise viral replication, leading to a defect;[32,33] however, it is important to note that viable viruses can still be produced despite its removal.[34,35] Furthermore, genome sequencing of SARS-CoV-2 variants during the COVID-19 pandemic revealed sites of positive selection in NSP2, indicating the adaptation of humans as a specific host following successful zoonotic co-transmission.[36] Importantly, the observed inability to rescue this defect by expressing NSP2 from an alternative genomic site highlights the indispensable role of the timing of NSP2 expression.[37,38] Despite the recent decrease in infected cases, the possibility of further waves of infection is worrying. However, there is also optimism that these potential outbreaks can be prevented or reduced with careful measures and surveillance.[25]

The studies of many researchers have been realized on the SARS-CoV-2 replication mechanism, pathogenicity, and therapeutic strategies. The aim of this study was to provide information about virus mutation, which has important implications for disease progression and the development of drugs or vaccines. In order to achieve this aim, the Open Reading Frame 1ab (ORF1ab) of SARS-CoV-2 was analyzed to evaluate the mutations caused by selection pressure on the virus and their impact on viral protein stability to infect human hosts, thereby promoting epidemic spread.

Materials and Methods.

Participants and Study Design. 100 COVID-19 patients were selected for the study from June to September 2022 at Shafa Hospital, affiliated with Kerman University of Medical Science, Kerman, Iran. The inclusion criteria for the study involved patients who satisfied the diagnostic standard for COVID-19.[39] The other inclusion criteria for study participants encompass the following conditions: 1) purification kit (Qiagen GmbH, Germany). After purification, it is sent to Baseline 1 (Gemini) company for sequencing via the Applied Biosystems 3730xl DNA Analyzer. Of the total sequences submitted, 90 Confirmation of SARS-CoV-2 infection through a throat swab with a cycle threshold (Ct) value less than 24, 2) Manifestation of clinical symptoms such as chest pain, cough with bloody or purulent sputum, diarrhea, dehydration, vomiting, and dyspnea, 3) Absence of reported symptoms associated with underlying medical conditions, and 4) Non-receipt of any of the existing COVID-19 vaccinations. A structured questionnaire was obtained for demographic information, medical history,

breath number, and temperature. Before participation, all individuals provided written informed consent, and the Ethics Committee of Kerman University of Medical Sciences approved the study (IR.KMU.REC.1402.024).

Samples. All patients underwent nasopharyngeal sample collection through the use of a specific swab. Samples are transferred to viral transfer media (VTM) and then translocated by a cool box.

Extraction of RNA. RNA isolation kit (Product no: 11856022001, Roje) was utilized to extract viral RNA from oropharynx/nasopharynx samples following the manufacturer's instructions. The concentration and purity of the extracted RNA were evaluated by measuring 1 µl of each sample with NanoDrop™ 2000 (Thermo Scientific, USA). Also, RNA integrity was assessed for its quality control by running 4 µl of the extracted RNA along with 2 µl Loading Day on 2% agarose gel.

Sequencing of NSP2. The extracted RNA samples were converted to complementary DNA (cDNA) using cDNA synthesis kits (Yekta-Tajhiz, Iran) according to the manufacturer's instructions. The sequencing of NSP2 of the ORF1ab gene was performed on 90 confirmed cases of COVID-19. The NCBI database and AlleleID software were used to extract the sequence of NSP2 and the design of specific primers for the target region, respectively. Conventional PCR techniques with Eppendorf Mastercycler were used to amplify the NSP2. PCR conditions included 1X PCR buffer, 300 - 400 ng of template cDNA, 1 mM MgCl2, 100 mM deoxynucleotide triphosphates (dNTPs), 10 pmol of each primer, and 0.5 U of Taq polymerase in a total volume of 25 µL. The PCR thermal profile was 95°C for 5 min, followed by 40 cycles of 95°C for 45 second, 59.4°C for 45 second, 72°C for 35 second, and a final extension for 5 min at 72°C. The primer sequence and annealing temperatures used for NSP2 amplification have been shown in **Table 1**.

For band detection, the products of the PCR amplification were subjected to electrophoresis on a 1.5% agarose gel using a 100 bp molecular weight marker. The bands corresponding to the 970bp studied region were located and excised from the gel for purification through the MinElute electrophoresis band results were clear and perfect for further analysis, including CLC6 and Clustal Omega for aligning and supplementing to obtain the sequence. The sequences were cross-referenced with existing databases to validate the results using the BLAST online search tool at www.ncbi.nlm.nih.gov. Furthermore, the sequences were compared to the reference strain Wuhan-Hu to identify any related mutations by utilizing Clustal Omega.

Variations of nucleotide. We conducted multiple sequence alignments to identify any nucleotide variations by using Clustal Omega.[40] The reference genome was the Whuhan-Hu-1 strain sequence, with GenBank accession number 045512. Clustal Omega's MVIEW program was used to analyze the alignment file.[41]

Variations of amino acid. Each protein's multiple sequence alignment was analyzed using MVIEW after being aligned again with Clustal Omega. In addition, amino acid variation was detected by comparing it to the reference strain protein.

The impacts of genetic mutations. Different prediction tools were employed to examine missense mutations' stability change and structural consequences. Specifically, I-mutant was utilized to determine structure stability.[26] Furthermore, Mutpred2 was employed to predict the molecular consequences and functional impact of missense mutations.[42]

Molecular Dynamic Simulations. Molecular dynamic (MD) simulations were performed using GROMACS96 43a1 program with CHARMM27 force field.[43] Each system was solvated with TIP3P water with a minimal distance of 1.0 nm between the solute and the wall of the dodecahedron box. Ionization states were assigned to titratable residues corresponding to the pH 7.0 condition. A proper amount of Na and Cl ions was added instead of water molecules to imitate an ionic strength of 0.15 M. The system was then energy minimized using the steepest descent algorithm with an initial step size of 0.01 nm for a maximum force of 1000 kJ/mol/nm and a maximum of 50,000 steps. Then, a 100-ps-long unconstrained equilibration MD simulation was done at a constant temperature (300 K) and pressure using Berendsen and Parrinello–Rahman coupling methods. Pressure coupling was performed using a reference pressure of 1.0 bar and a time constant of 1.0 ps. Finally, a 50-ns-long production MD simulation was performed at a constant temperature of 300 K, maintained by the v-rescale thermostat.

Phylogenetic analysis. Phylogenetic and sequence analyses were conducted to investigate the evolutionary relationships between different isolates. Of the 90 samples with clear and complete sequencing results, 39 were selected for constructing the phylogenetic tree. The remaining cases were excluded either due to the similarity in their mutations or the absence of mutations in their sequences, which would hinder the construction of the phylogenetic tree. Reference sequences from the Wuhan strain and concerning variants, including B.1.1.617.2, B.1.1.7, B.1.1.529, B.1.351, and P.1, were used for analysis and phylogenetic tree construction.

Table 1. The NSP2-specific primer sequences utilized for RT-PCR.

Primer sequence (5′ -3′)	Annealing temperature (°C)	Position	Product size
FW: AATGCTGGTATTGTTGGT	58.1	1228-2310	970
RW: GTTGACGATGACTTGGTTA			

Clustering of sequences was performed with MEGA 11software, followed by inference of evolutionary history using the Maximum Likelihood method with ~1000 bootstrap iterations.

Results.

Demographic data. Out of the total submitted sequences, 90 results for NSP2 having clear and complete sequencing were included. The nucleic acid sequences were obtained from 48 males and 42 females, and patients' mean ages were 51.08.

The SARS-CoV-2 variant classification. The SARS-CoV-2 variant classification refers to categorizing different strains or variants of the SARS-CoV-2 virus based on specific genetic mutations or changes in its genome. These variants are identified through genomic sequencing and analysis, which helps understand the spread, evolution, and potential impact of different viral lineages. The classification typically involves assigning names or designations to different variants based on their specific mutations, such as Alpha/B.1.1.7, Beta/B.1.351, Gamma/P.1, Delta/B.1.1.617.2, Omicron/B.1.1.529, and so on. The variant classification provides important information for monitoring the virus's global spread assessing its transmissibility, virulence, and potential impact on diagnostics, therapeutics, and vaccines. It helps researchers, public health authorities, and healthcare professionals to track and respond to the emergence and prevalence of different SARS-CoV-2 variants.

Mutations found in SARS-CoV-2 isolates from Kerman. All 90 Kerman isolates' analysis showed 17 Single-nucleotide polymorphisms (SNP) in the NSP2. A deletion was also identified for NSP2 isolate (Table 2). Out of 17 mutations, there were 8 missense mutations in positions 207, 224, 262, 265, 337, 348, 392, and 431, whereas the remaining mutations were synonymous (**Table 3**).

Table 2. Demographic information of the study patients

Clinical characteristics	Patients (n = 90)	Demographic data	Patients (n = 90)
Dyspnea		**Age**	51.08 ± 3.6
Present	27 (30%)		
Absent	63 (70%)		
Diarrhea		**BMI**	
Present	25 (27.7%)	<18.5	3 (3.3%)
Absent	65 (72.3%)	18.5-24.9	24 (26.6%)
Vomiting		25-29.9	28 (31.1%)
Present	12 (13.3%)	>30	35 (38.8%)
Absent	78 (86.6%)		
Chest pain		**Smoking**	
Present	28 (31.1%)	Yes	47 (52.2%)
Absent	62 (68.8%)	No	43 (47.8)
Purulent sputum cough			
Present	13 (14.4%)		
Absent	77 (85.6%)		
Temperature		**Gender**	
Present	69 (76.6%)	Male	48 (53.3%)
Absent	21 (23.4%)	Female	42 (46.6%)

Table 3. The type of mutation and the position of the mutations detected in amino acid and nucleotide in NSP2.

Number	Nucleotide substitution	Protein	Amino acid substitution	Mutation type
1.	885: G >A	ORF1ab	R207C	Missense
2.	936: C > T	ORF1ab	T224I	Missense
3.	1005: C > A	ORF1ab	-	Synonymous
4.	1019: C > T	ORF1ab	-	Synonymous
5.	1030: C > G	ORF1ab	-	Synonymous
6.	1045: G > T	ORF1ab	G262V	Missense
7.	1059: C > T	ORF1ab	T265I	Missense
8.	1167: C > T	ORF1ab	-	Synonymous
9.	1184-1214: Deletion	ORF1ab	-	Deletion
10.	1218: G > T	ORF1ab	-	Synonymous
11.	1276: T > G	ORF1ab	K337D	Missense
12.	1291: G > C	ORF1ab	-	Synonymous
13.	1308: A > G	ORF1ab	N348S	Missense
14.	1441:C > T	ORF1ab	G392D	Missense
15.	1558: G > A	ORF1ab	I431M	Missense
16.	1749: A > C	ORF1ab	-	Synonymous
17.	1867: T > G	ORF1ab	-	Synonymous

Table 4. Predicting the stability of NSP's structure under the influence of missense mutations.

	Amino acid substitution	SVM2 Prediction Effect	DDG (kcal/mol)
nsp2	R207C	Decrease	-0.81
nsp2	T224I	Decrease	-0.54
nsp2	G262V	Decrease	-0.93
nsp2	T265I	Decrease	-0.79
nsp2	K337D	Increase	-0.19
nsp2	N348S	Decrease	-0.52
nsp2	G392D	Increase	-0.25
nsp2	I431M	Decrease	-0.47

Abbreviation: SVM2, support vector machine for detecting small genomic structural variations using high-throughput single-genome resequencing data.; DDG predicted free energy change.

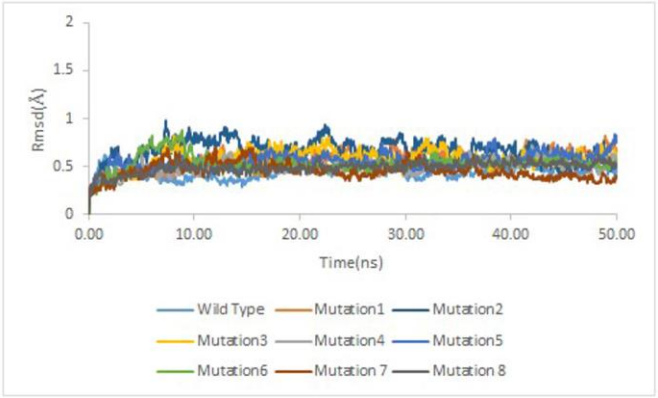

Figure 1. Root mean square deviation (RMSD) in nm was depicted for wild-type (WT) (Blue), R207C (Orange), T224I (Dark blue), G262V (Yellow), T265I (Light gray), K337D (Light blue), N348S (Green), G392D (Brown), I431M (Dark gray) NSP2s during the 50-ns molecular dynamic (MD) simulations.

Effects of various mutations in NSP2. Six of the eight missense mutations identified in NSP2 indicated decreased structural stability. In addition, two remaining mutations have an increase in stability of structural (**Table 4**).

Root mean square deviation. Root mean square deviation (RMSD) was also used to investigate the stability of the structures.further It was assessed during the 50-ns MD simulation runs for WT nsp2 and R207C, T224I, G262V, T265I, K337D, N348S, G392D, I431M nsp2 systems. Unlike other mutations, the RMSD in the K337D (Purple) and the G392D (Brown) mutation were lower compared to the wild variant, indicating that these mutations were more structurally stable (**Figure 1**).

Anticipation and verification of homology model predictions. Nine models were produced for NSP2 using the PDB ID 7MSX as a template: the eight models were for the Kerman isolate, and the remaining one was for the reference strain. Mutant models 1 to 8 are designed for R207C, T224I, G262V, T265I, K337D, N348S, G392D, and I431M mutations, respectively. The reliability of these 8 models was assessed with validation assessment scores, which were similar to the template (**Table 5**).

Phylogenetic analysis. In this study, we compared the NSP2 sequencing results to globally registered concern variants and provided detailed specifications in **Figure 2**. According to our study, the predominant SARS-CoV-2 viruses circulating were comparable to the Delta/B.1.617.2 variant (**Figure 2**). In addition, we found that the NSP2 variant of concern (VOCs) prevalence with Pangolin Lineages Delta was 89.74%

Table 5. Reliability assessment of different mutant Models.

Mutant	Rampage Score		ERRATA Score
	Favored	Allowed	
Mutant model 1	92.4%	5.6%	83.4%
Mutant model 2	92.7%	5.4%	82.9%
Mutant model 3	91.3%	6.8%	82.1%
Mutant model 4	88.9%	7.3%	83.1%
Mutant model 5	90.6%	6.9%	80.3%
Mutant model 6	93.2%	4.9%	78.9%
Mutant model 7	92.4%	5.9%	84.9%
Mutant model 8	91.7%	6.3%	81.2%
Template	96.1%	3.8%	82.5%
Wild type	92.7%	5.8%	82.4%

Rampage program was used to calculate the amino acid assembly point percentage. ERRATE score showed the quality of protein structure.

while Omicron/B.1.1.529 accounted for 5.1% (**Figure 2**).

Discussion. The COVID-19 pandemic has emerged as a worldwide crisis.[24] Viral genome mutations and subsequent modification of viral proteins are common ways for viruses to escape from the immune system response and survive within the host for extended periods.[34,37] Although cases of the disease have decreased recently, there are still concerns about the possibility of further waves of infection.[36,38] This study analyzed the sequencing of SARS-CoV-2 in Kerman, Iran, and identified variations that could offer insights into the virus's pathogenesis, genetic diversity of NSP2, and the potential impact of mutations. A total of 17 mutations were detected; 8 mutations were related to missense mutation, including R207C, T224I, G262V, T265I, K337D, N348S, G392D, I431M, and N348S, and one mutation was related to deletion.

The NSP2 contains two functional clusters, one containing three proteins involved in vesicle transport.[44] In our study, some mutations, such as V1883T and R207C, occurred in this cluster, which may result in the effective virus release from the endosome. The remaining cluster comprises eight proteins associated with ribosome assembly and has the potential to obstruct the transcription and translation process of human mRNAs.[45] Other mutations in this cluster include T224I, G262V, and T265I, which may alter the pathogenic pathway of SARS-CoV-2 by interfering with proteins or cellular signaling. A prior investigation has demonstrated that NSP2 may impact calcium homeostasis, and alterations such as N348S and I431M mutations could potentially modify its function. This modification is significant because apoptosis is a crucial mechanism for host cell defense against SARS-CoV-2 infection.[46] According to the identification of these mutations in our study, it is possible that the variants

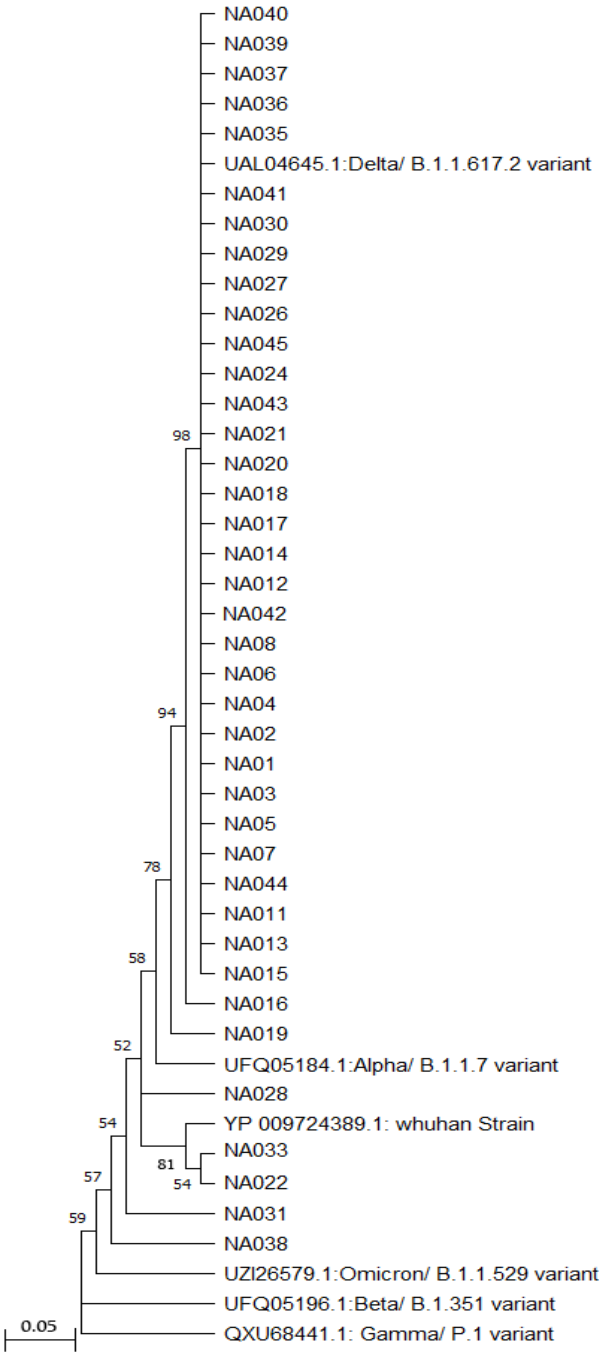

Figure 2. Phylogenetic tree. The evolutionary NSP2 was depicted through a phylogenetic tree. The tree was constructed using MEGA software, the Maximum Likelihood method (~1000 bootstrap), and the Tamura model.

caused by mutations can alter cell apoptosis by altering calcium homeostasis. On the other hand, some RNA-binding proteins, such as STAU2, play an anti-apoptotic role in DNA replication and genome integrity maintenance.[47] The binding of SARS-CoV-2 NSP2 protein to STAU2 may inhibit its anti-apoptotic function,[48] and the mutations in NSP2 may affect this interaction.

The NSP2 can interact with some of the guanine nucleotide exchange factors such as RAP1GDS1, and by changing the activity of some small GTPases, it might aggravate or alleviate lung inflammation during SARS-2 infection,[14,49] which is consistent with the mutations identified in our study. Many organelles, including endosomes, lysosomes, and exosomes, have protein complexes such as V-ATPase in their membranes, which transfer protons to the organelle to maintain the acidic environment. During SARS-CoV-2 infection, researchers have demonstrated that NSP2 interacts with some subunits of V-ATPase and is involved in transporting substances such as $Ca2^{+}$.[50] Therefore, some mutations that occurred in NSP2 can change the interaction between it and V-ATPase and may participate in induced during endocytosis.

In addition, NSP2 could be considered one of the targets of laboratory diagnosis for SARS-CoV-2 by rapid and real-time reverse transcriptase-polymerase chain reaction (rRT-PCR). In their study, Yip et al. identified a 154 nucleotide fragment as a conserved region for detecting SARS-CoV-2.51. Therefore, any mutation at the 1867 position in our study could potentially fail to identify SARS-CoV-2 patients. Continuously monitoring mutations will be crucial to track the virus's spread among individuals and across different regions.

Another mutation was responsible for deletion. Large deletion mutations can cause a defect in the production and function of the desired protein. In this regard, some scientists have suggested that large deletions in OFR1ab may not encode the target protein.[52] Large deletion mutations have also been found in the studies of other scientists; for instance, an 80-nucleotide deletion in ORF7a was also reported in a study conducted in Arizona.[53]

In concordance with our study, Banerjee et al. identified a prevalent mutation, specifically T265I, within the NSP2 gene. Their research spanned 31 different states across the United States, involving the examination of 867 complete protein sequences of ORF1ab. Notably, they found that among the genes comprising the ORF1ab region, which constitutes approximately two-thirds of the SARS-CoV-2 genome, the T265I mutation exhibited the highest incidence, accounting for around 50%. These findings are consistent with our investigation, as the NSP gene is known to influence mitochondrial function, manage cellular stress, and modulate host cell survival signaling pathways by interacting with PHB and PHB2 proteins within the host organism. Consequently, the T265I mutation in this gene may confer advantages to the virus in these processes.[31] In alignment with the findings of Koyama et al., our study has also highlighted the significance of the G392D mutation in the context of SARS-CoV-2 evolution and its potential impact on viral stability. Koyama et al.'s comprehensive analysis of over 10,000 SARS-CoV-2 genomes from diverse geographical regions revealed a spectrum of genetic variants, including the G392D mutation.[54]

Conclusions. Our study delves into the intricate realm of SARS-CoV-2 evolution, shedding light on crucial aspects contributing to its adaptability and potential impact on public health. Notably, we have elucidated a spectrum of variations within NSP2, uncovering a nuanced narrative of viral stability. While some genetic variations may undermine NSP2 stability, intriguingly, others, such as K337D and G392D, appear to bolster it. This dualistic insight into NSP2's stability diversification adds a novel layer to our understanding of the virus's adaptive mechanisms. Our work also extends beyond the realm of basic research. The homology models we have meticulously designed to elucidate the structural consequences of NSP2 mutations provide a valuable resource for future studies aiming to decipher the functional implications of these genetic changes. Importantly, these models align with the Wuhan strain and reveal a striking resemblance to the Delta variant, underscoring the relevance of our findings in the context of contemporary viral evolution.

Furthermore, our findings carry significant implications for public health. We emphasize the pressing need for continuous surveillance of genomic variations within SARS-CoV-2, especially in the face of emerging variants like Delta, to inform the development of effective treatment strategies and updated vaccines. In conclusion, our research offers a comprehensive and nuanced perspective on SARS-CoV-2 evolution, accentuating our unique insights into NSP2 variations, their structural implications, and their relevance in contemporary viral landscapes. This multi-faceted contribution advances our understanding of the virus and provides a foundation for future investigations to combat the ongoing global health challenge.

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Author Contributions. NA, ZG designed the study, and NA Analyzed the data. NA and MN Contributed new methods or models. NA and ZG wrote the paper. MRZR, NA, and MN reviewed and finalized the manuscript. All authors read and approved the final manuscript.

Ethics approval. This study was performed in line with the principles of the Declaration of Helsinki. Approval

was granted by the Ethics Committee of Kerman University of Medical Science (IR.KMU.REC.1402.024).

Consent to participate. Informed consent was obtained from all individual participants included in the study.

Consent to publish. The authors affirm that human research participants provided informed consent for publication.

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