Original Article

The Study of Mutations and Phylogenetics of the SARS-CoV-2 Spike Gene in Population from Tehran Province

Mohamadzadeh, M[1], Keyvani, H[2], Latifi, AM[1], Keyvani, F[3], Azizi, MR[4], Ranjbar, MM[5*], Karimi GH[5]

1. *Applied Biotechnology Research Center, Baqiyatallah University of Medical Science, Tehran, Iran.*
2. *Department of Virology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran.*
3. *Department of Electrical and Computer Engineering, Faculty of Engineering, University of Waterloo, Waterloo, ON N2L, Canada.*
4. *Department of Microbiology, Faculty of Sciences, Islamic Azad University, Karaj Branch, Karaj, Iran.*
5. *Razi Vaccine and Sera research institute, Agricultural Research, Education & Extension Organization (AREEO), Karaj, Iran.*

How to cite this article: **Keyvani H, Latifi AM, Mohamadzadeh M, Keyvani F, Azizi MR, Ranjbar MM, Karimi GH. The Study of Mutations and Phylogenetics of the SARS-CoV-2 Spike Gene in Population from Tehran Province. Archives of Razi Institute. 2025;80(1):185-192.** DOI: 10.32592/ARI.2025.80.1.185

Article Info:
Received: 1 October 2024
Accepted: 1 December 2024
Published: 28 February 2025

Corresponding Author's E-Mail:
mm.ranjbar.phd@gmail.com

ABSTRACT

In December 2019, an outbreak of pneumonia of unknown etiology was reported in Wuhan, China. The virus, known as SARS-CoV-2, is contagious and infects the lower respiratory tract. Since various coherent research needs to be conducted in Iran to detect mutations in the SARS-CoV-2 S gene, the present study was conducted to determine the sequence, mutation pattern, and phylogenetic evaluation of this gene. To this end, 120 positive samples were included in the study to evaluate the complete S gene sequence by Reverse transcriptase-PCR.Subsequent to the sequencing process, the gene assembly, blasting, mutation analysis, and phylogenetic analysis were performed using MEGA-X.A total of 161 mutations were observed in the S gene sequences of Iran. The results of the phylogenetic tree showed that all the S gene sequences of Iranian samples were divergent from the Wuhan strain and had the most similarity to it and also alpha variants. 161 nonsynonymous variations were found along the complete coding S gene with a high frequency of A262T, D614G, and P863H, which were embedded in HVR1, HVR2, and HVR3, respectively. The majority of highly variable fragments have been identified in the loop secondary structure of protein. In the present study, the predominant variants (predominantly alpha variants) and mutations were observed to be in parallel with the evolution of the virus and its fitness. A comprehensive overview of the genetic mutation of the first three waves of SARS-CoV-2 in Iran was provided, which could be used to make significant decisions and take effective measures in future pandemics to develop vaccines, kits and effective therapeutics.

Keywords: SARS-CoV-2, S Gene, Mutation, Phylogenetic Analysis.

1. Introduction

At the conclusion of 2019, an outbreak of severe acute respiratory syndrome (SARS) was reported from Wuhan, China, which subsequently spread rapidly on a global scale. The causative agent was identified as a novel coronavirus (2019-nCov). Subsequently, the virus was designated severe acute respiratory syndrome coronavirus-2 (SARS-Cov2). The high transmissibility of SARS-CoV-2 resulted in the declaration of a pandemic by the World Health Organization (WHO) in March 2020 (1). As of now, the WHO has reported more than 251,788,329 confirmed cases and 5,077,907 deaths (2). The virus belongs to the Nidovirales order, coronaviridae family, coronavirinae subfamily and Betacoronavirus genus (3). It possesses a single-stranded positive-sense RNA genome of 29,891 bp in size, as demonstrated in Figure 1. The genome organisation is as follows: the spike protein, as a surface glycoprotein, is responsible for receptor binding, and the spike protein composes two subunits: S1 and S2, with the function of receptor binding and membrane fusion, respectively (4).The S1 subunit can bind to angiotensin-converting enzyme-2 (ACE-2) and other alternative receptors, such as CD147 and neuropilin, on the host cell and mediates the cell entry (4, 5). It is well established that during the replication of coronaviruses, a multitude of mutations can occur, the majority of which are deleterious and neutral. However, it is noteworthy that certain mutations have the capacity to exacerbate the severity, infectivity and transmissibility of the virus (6). Indeed, mutations within the receptor-binding domain (RBD) of the S1 subunit have been shown to disrupt species barriers, thereby facilitating viral transmission between animals and humans (7). Evidence indicates that certain variations in the SARS-CoV-2 S gene can not only alter the viral antigenic phenotype, but also have a significant impact on viral infectivity, immune evasion, and pathogenicity (8). For instance, a nonsynonymous substitution at position 614 (D614G) has been observed to enhance viral replication and transmission, though there is no clear association with increased severity of infection (9). The Global Influenza Surveillance and Response System (GISRS) has identified 21 distinct clades for SARS-CoV-2 based on the mutations across the whole genome of the virus (https://nextstrain.org). Conversely, the WHO has recently adopted a novel nomenclature system for variants of concern, utilising the Greek alphabet. These variants are categorised as Alpha (lineage B.1.1.7), Beta (lineage B.1.3 51), Gamma (lineage P.1), Delta (lineage B.1.617.2), and Omicron (lineage B.1.1.529) (https://cov-lineages.org).From July to November 2021, Delta variants (21I and 21J) were the most prevalent circulating variants worldwide, causing concern even among fully vaccinated individuals. In late November 2021, a new variant designated Omicron was identified by the WHO, which was predicted to spread rapidly. Variations in the surface glycoprotein have the capacity to modify infectivity and immune responses. The present study therefore sought to investigate the complete coding sequence of the S gene in circulation among the Iranian population, with a view to ascertaining its genetic variations.

2. Materials and Methods

2.1. Sample Collection

In the period between February and November 2020, a total of 120 nasal and/or oropharyngeal swabs were collected from patients referred to the Keyvan Virology Laboratory in Tehran. The samples that were positive for the SARS-CoV-2 test were included in the study.

2.2. Viral RNA Extraction and Complementary DNA (cDNA) Synthesis

The isolation of viral RNA from the samples was conducted in accordance with the stipulated instructions provided by the manufacturer of the LabPrep TM Viral DNA/RNA Mini Kit. The subsequent synthesis of cDNA was facilitated by the biotech rabbit GmbH kit (Berlin, Germany), utilizing a total reaction volume of 20 µl and the following components:

2 µl dNTP mix (10 mM for each), 0.5 µl RNase inhibitor, 0.5 µl oligo dT primer, 1 µl random hexamer primer, 4 µl 5x reverse transcriptase buffer biotech rabbit kit, 10 µl RNA template, 1 µl Moloney-Murine Leukemia Virus (MMuLV) reverse transcriptase, 1 µl RNase-free water. The RT-PCR reaction profile was set up at 30°C for 10 min, 55°C for 40 min, and 99°C for 5 min. The complete coding sequence of the S gene was amplified using primers and a PCR profile, the details of which can be found in Table 1. In addition, the β-globin gene was utilized to ensure the quality of the samples.

2.3. Bioinformatic Analysis

The amplified sequences were verified by 1% agarose gel electrophoresis and subsequently sent for Sanger sequencing. The amplicons that were successfully sequenced were analysed using BioEdit (10) and then subjected to blast searches using BLASTn, BLASTp and MEGA X (11) with reference sequences (NC_045512, MZ314997, MZ314998, MZ611965 and OK091006). The construction of the phylogenetic tree was performed using the neighbour-joining method with the Kimura 2-parameter model and a bootstrap value of 1000, implemented in MEGA X.To investigate the variations present in the understudied sequences, these were analysed as either being embedded in hypervariable regions (HVRs) or not. To this end, Shannon entropy analysis was employed, with a threshold of 0.3, thus revealing three regions: 1) HVRs exhibiting a higher degree than the threshold, 2) semi-HVRs situated near the threshold, and 3) variable regions falling beneath the threshold. The 3D structure of the spike glycoprotein model was modelled using the SWISS-MODEL online server (https://swissmodel.expasy.org/interactive), with energy minimisation conducted using SPDB viewer software. Visual analysis and editing was performed using Chimera UCSF software.

Table 1. Primers and thermal cycling profiles.

Primer name	5'- 3' sequences	PCR product Length	Thermocycler program	Ref
25-Forward **25-Reverse**	CTTGGAGGTTCCGTGGCTAT AAACCCTGAGGGAGATCACG	1077 bp	Primary denaturation: 94 °C, 150 s Denaturation: 94 °C, 40 s Annealing: 59 °C, 55 s x39 Extension: 72 °C, 128 s Final extension: 72 °C, 600 s	(3)
26-Forward **26-Reverse**	TATCTTGGCAAACCACGCGA ACCAGCTGTCCAACCTGAAG	1057 bp	Primary denaturation: 94 °C, 210 s Denaturation: 94 °C, 40 s Annealing: 58 °C, 45 s x39 Extension: 72 °C, 120 s Final extension: 72 °C, 420 s	(3)
27-Forward **27-Reverse**	CCCTCAGGGTTTTTCGGCTT CTGTGGATCACGGACAGCAT	1093 bp	Primary denaturation: 94 °C, 180 s Denaturation: 94 °C, 40 s Annealing: 60 °C, 48 s x39 Extension: 72 °C, 150 s Final extension: 72 °C, 420 s	(3)
28-Forward **28-Reverse**	CCAGCAACTGTTTGTGGACC GTGGCAAAACAGTAAGGCCG	1027 bp	Primary denaturation: 94 °C, 180 s Denaturation: 94 °C, 40 s Annealing: 60 °C, 48 s x39 Extension: 72 °C, 135 s Final extension: 72 °C, 480 s	(3)
29-Forward **29-Reverse**	ACTTGCAGATGCTGGCTTCA CTCATTGAGGCGGTCAATTTCT	1084 bp	Primary denaturation: 95 °C, 135 s Denaturation: 94 °C, 40 s Annealing: 57 °C, 58 s x39 Extension: 72 °C, 120 s Final extension: 72 °C, 540 s	(3)
30-Forward **30-Reverse**	TGATTTAGGTGACATCTCTGGCA ACAACTCCGGATGAACCGTC	1106 bp	Primary denaturation: 95 °C, 180 s Denaturation: 94 °C, 40 s Annealing: 59 °C, 50 s x39 Extension: 72 °C, 135 s Final extension: 72 °C, 420 s	(3)

3. Results

3.1. Mutations

In the present study, the complete coding sequence of the S gene was successfully determined by means of sequencing and translation to amino acid sequences for 117 of the 120 samples enrolled. This was achieved using the online tool Basic Local Alignment Search Tool (BLASTx).A total of 161 nonsynonymous substitutions were observed in understudied amino acid sequences (Table 2). The most prevalent variations included V11I (n=17), T22I (n=11), K77N (n=12), D111N (n=15), A262T (n=42), D614G (n=45), and P863H (n=23). Furthermore, 3D structural analysis demonstrated that the majority of variations within this study, particularly those that were frequently substituted, such as A262T, D614G, and P863H, were located in SHVR1 (amino acid positions 13-25), HVR1 (amino acid positions 255-265), SHVR2 (amino acid positions 485-496), HVR2 (amino acid positions 613-620), and HVR3 (amino acid positions 857-865) (data not displayed).

3.2. Shannon Entropy and 3D Structure Analysis

The Shannon entropy variation analysis indicates the presence of three HVRs in the spike glycoprotein of SARS-CoV-2, including HVR1 in the N-terminal domain at positions 255-265, HVR2 in the S1 subunit at positions 613-620, and HVR3 in the S2 subunit at positions 857-866. HVR2 was identified as the most variable region among understudied sequences. In addition, three semi-HVRs were identified: amino acids 13 to 25 in the N-terminal domain, 485-495 in the receptor binding domain, and 540-548 in the S1 subunit.As anticipated, the C-terminus of the spike glycoprotein and amino acids surrounding the cleavage site (positions 672 to 698) exhibited the lowest variability. The Shannon entropy map and the three-dimensional structure of the spike glycoprotein amino acid sequences with different variable regions related to Iranian samples are illustrated in Figures 1 and 2.

3.3. Phylogenetic Analysis

The neighbour-joining method was employed to construct the tree, with the software Mega 10 utilised for this purpose. As illustrated in Figure 3, all understudied sequences diverged from the Wuhan isolate, with some sequences demonstrating clustering with alpha variants, such as MW090904 and MW040523. The isolates MW040527, MW09087 9, MW090877, MW136260, MW045462, MW090866, MW136350, and MW136262 depicted most divergency from the Wuhan isolate, suggesting the initiation of further changes.A deeper

Table 2. Mutations and their frequencies in the sequenced samples.

	2	9	11	13	14	18	21	22	30	34	37	40	42	44	47	59	65	74	77	78	95	97	106	111	115	123	136	141	149	151	156
Ref	F	P	V	S	Q	L	R	T	N	R	Y	D	V	R	V	F	F	N	K	R	T	K	F	D	Q	A	C	H	N	S	E
Iranian samples	L	Q	I	N/R	H	P	K	I	I	H	N	E	L/I	K/I	I	I	I	H	N	K	I	N	L	N	H	T	W	R	H	G	D
counts	2	1	17	2/6	5	2	3	11	2	2	3	1	2/4	7/2	7	2	2	2	12	2	1	2	2	15	4	2	2	2	1	1	5

	158	159	161	162	169	173	181	185	187	190	191	193	194	198	199	200	206	207	210	211	212	213	215	216	220	222	224
Ref	R	V	S	S	E	Q	G	N	K	R	E	V	F	D	G	Y	K	H	T	N	L	V	D	L	F	A	E
Iranian samples	L	I	F	I	Q	H	R	I	N/Q	S	D	L	L	E	R	N	N	L	S	Y/H	S	L/G	H	H	V	T/V/P	K
counts	2	3	2	2	2	4	1	2	2/3	3	2	2	2	2	2	3	2	3	2	1/1	1	1/1	1	1	1	1/1/3	8

	225	226	227	228	229	231	234	235	240	247	262	271	288	302	407	408	411	412	427	440	448	463	485	487	488	510	511	512
Ref	P	L	V	D	L	I	N	I	T	S	A	Q	A	T	V	R	A	P	D	N	N	P	G	N	C	V	V	V
Iranian samples	S/R/T	I	I/M/A	I/E/N/Y	F	L	S	V	S	N	T	H	P	A	L	T	T	S	N	H	K	L	S	I	R	E	I	I
counts	1	1	2/1/1	1/2/3/1	2	4	2	1	2	3	42	2	3	2	3	2	2	2	2	2	2	2	2	5	3	3	2	3

	520	522	534	535	535	536	568	614	620	636	648	652	653	655	658	661	669	672	675	677	684	686	688	702	705	710	716	726
Ref	A	A	V	K	N	N	D	D	V	Y	G	G	A	H	N	E	G	A	Q	Q	A	S	A	E	V	N	T	I
Iranian samples	S	P	G	V/E	E	K	H	G	F	F	R	R	P	Y	H	D	R	P	R	H	V	T	T/P	K	I/F	T	P	F
counts	1	4	2	2/2	2	2	2	45	2	2	4	3	3	2	3	3	5	5	7	8	2	2	2	3	4/2	2	2	2

	747	758	780	781	790	791	798	811	815	820	826	830	837	838	834	847	848	851	863	874	875	903	905	916	932	1019	1181
Ref	T	S	E	V	K	T	G	K	R	D	V	D	Y	G	D	R	D	C	P	T	S	A	R	L	G	R	K
Iranian samples	P	I/T	K	F	Q	P	A	N	K	N	G	N	N	A/D	G	K	N	W	H	P	F	G	S	F	A	K	Q
counts	2	3/2	2	3	3	2	2	2	3	5	3	3	2	2/2	3	4	4	4	23	3	3	2	2	3	2	2	2

analysis of the tree, based on topology and branches, resulted in the division of the tree into five main groups. The largest of these groups, designated Group 1, is further subdivided into Group 2. The most divergent sequence, characterised by its greater distance from the common ancestor of the sequences and the presence of more mutations and divergence, is MW045461. This sequence belongs to the first wave of the disease.The reference sequence is located in the first cluster, which is the largest cluster in Iran and contains the majority of the sequences. Cluster 5 is the smallest cluster and the sequences in it are more divergent and different than the other sequences and are closer to the common ancestor of the sequence.These sequences are MW045462 and MW090877, which are related to the second wave of corona virus infection in Iran, and an interesting point.Note the difference between these and other sequences, especially the existing sequences from the first wave of the disease. It is conceivable that these sequences may be the ancestors of certain Iranian sequences, and that other sequences may have arisen from them.

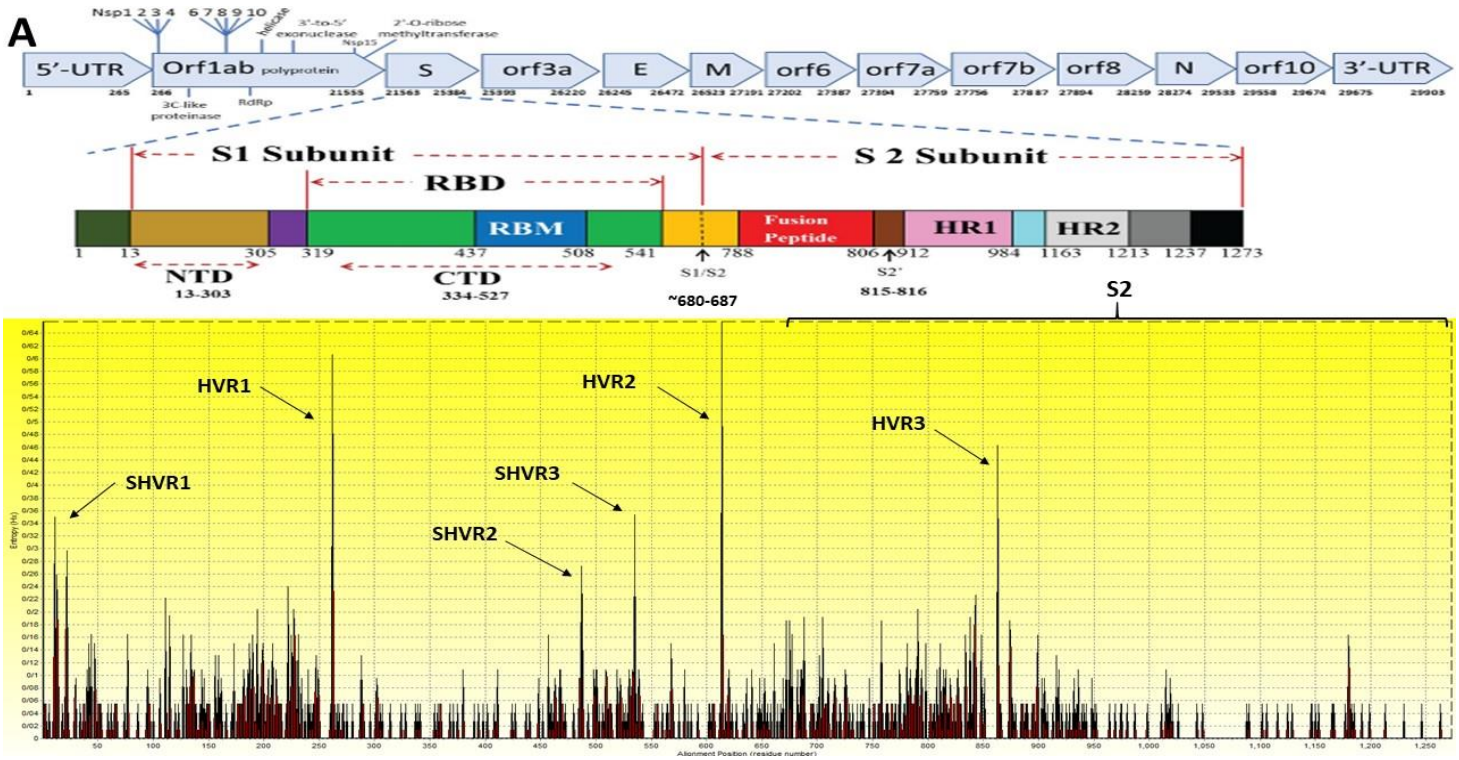

Figure 1. Shannon entropy of SARS-CoV-2 spike glycoprotein. The X and Y axes indicate amino acid positions and entropy, respectively. Bars near zero indicate the conserved sequences while bars greater than zero with greater height show amino acid positions with much variability, which show by arrows. Also, the S1, S2 subunits, and cleavage sites (positions 680-687) are displayed. HVR, hypervariable region; SHVR, Semi- hypervariable region.

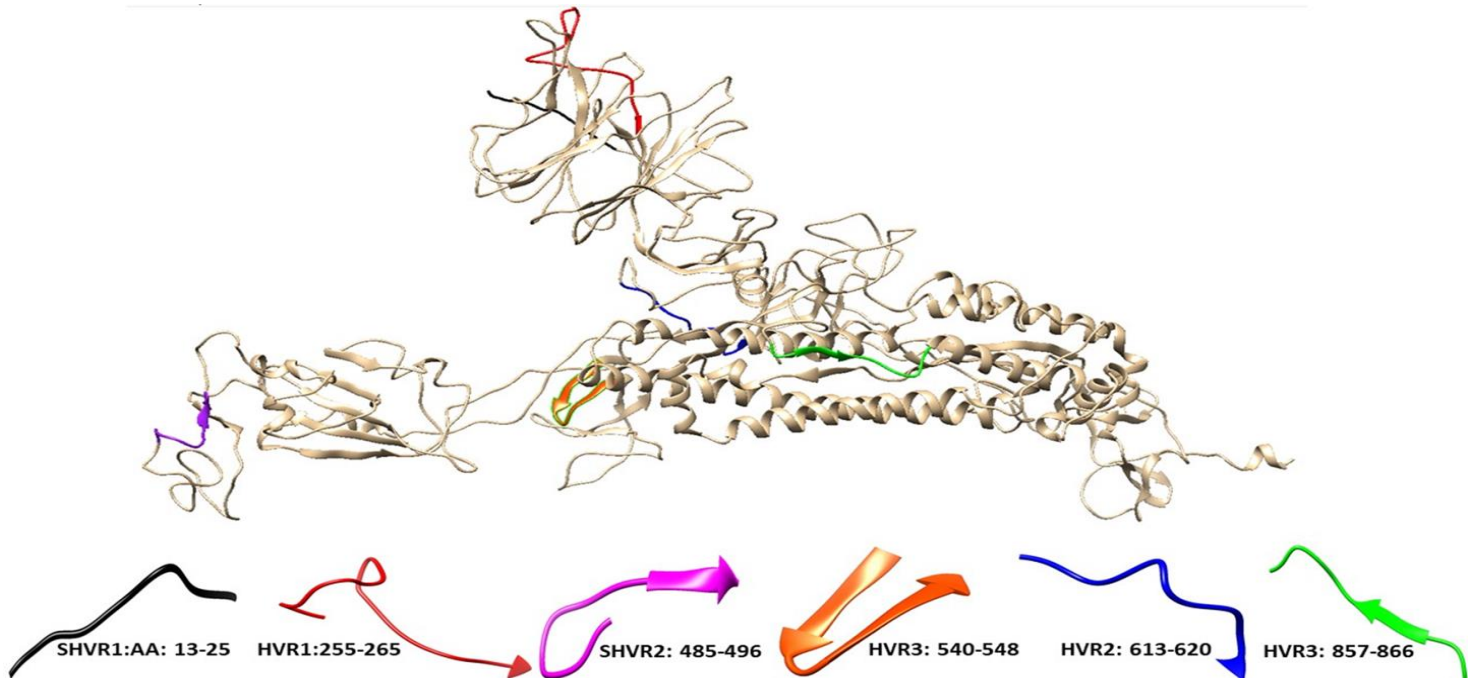

Figure 2. 3D structure of modeled Spike glycoprotein from Iran and mutation regions. Most of Highly variable fragments have been identified in loop secondary structure of protein.

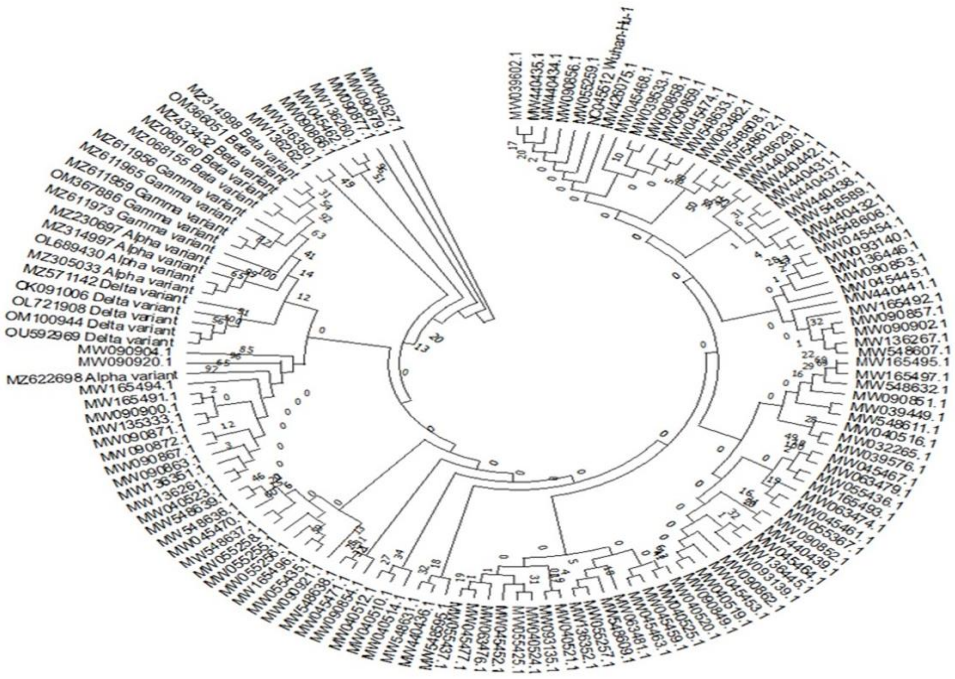

Figure 3. Phylogenetic tree of S gene from Iranian samples. The tree was drawn by using the neighbor-joining method, Kimura 2-parameter model with 1000 bootstrap replicates through Mega 10. The numbers above branches with values lower than 70 are hidden. The reference genomes are displayed with name of "NC045612 Wuhan-Hu-1" and remained accession numbers are responsible for isolates from this study. The Genbank accession numbers of all isolates from this study are gathered in Table 3.

Table 3. The accession numbers obtained from this study.

MT163712	MW032265	MW039449	MW039533	MW039576	MW039602
MW040510	MW040512	MW040514	MW040516	MW040519	MW040520
MW040521	MW040523	MW040524	MW040525	MW040527	MW045445
MW045452	MW045453	MW045454	MW045459	MW045461	MW045462
MW045463	MW045464	MW045467	MW045468	MW045470	MW045471
MW045474	MW045477	MW055255	MW055256	MW055257	MW055258
MW055259	MW055367	MW055425	MW055435	MW055436	MW055437
MW063474	MW063476	MW063479	MW063481	MW063482	MW090849
MW090851	MW090852	MW090853	MW090854	MW090856	MW090857
MW090858	MW090859	MW090862	MW090863	MW090867	MW090866
MW090871	MW090872	MW090877	MW090879	MW090900	MW090902
MW090904	MW090920	MW090921	MW093135	MW093139	MW093140
MW135333	MW136260	MW136261	MW136262	MW136267	MW136350
MW136351	MW136352	MW136445	MW136446	MW165491	MW165492
MW165493	MW165494	MW165495	MW165496	MW165497	MW426075
MW440431	MW440432	MW440435	MW440434	MW440436	MW440437
MW440438	MW440439	MW440440	MW440441	MW440442	MW548589
MW548595	MW548606	MW548607	MW548608	MW548609	MW548611
MW548612	MW548629	MW548631	MW548632	MW548633	MW548636
MW548637	MW548638	MW548639			

4. Discussion

The present study set out to determine the mutations and lineages of Iranian SARS-CoV-2 samples based on the S gene. Phylogenetic analysis showed that all understudied sequences were grouped with the Wuhan SARS-CoV-2 isolate and alpha variants, as had been expected. Indeed, until November 2020, alpha variants had been often circulating. 161 nonsynonymous substitutions were found in the understudied sequences. The most prevalent substitutions included V11I (n=17), T22I (n=11), K77N (n=12), D111N (n=15), and A262T (n=42) within the N-terminus domain, with V11I situated within the signal peptide. The most prevalent variants were found to be D614G (n=45), A262T (n=42), and P863H (n=23). The earliest documented samples of Delta variants were recorded in India in October 2020 (12), while the majority of our samples were collected in the mid-2020s, suggesting the emergence of this variant in Iran. A notable variation among the Delta variants was the D614G substitution, which was identified in 38.5% of understudied sequences. While this substitution increased the replication of SARS-CoV-2 in various cells that express the ACE-2 receptor on their surfaces, it did not affect the potency of neutralising antibodies. A total of six amino acid changes in and around the cleavage site S1/S2 were identified, including A672P, Q675R, Q677H, A684V, A686T, and A688T/P. The presence of basic amino acids, such as lysine and histidine, at cleavage sites, particularly at positions Q675R and Q677H, has been demonstrated to enhance transmissibility by facilitating viral entry through the pH-independent pathway (15). Consistent with the findings of this study, Eslami et al. reported that the P863H variation, located in the fusion peptide-heptad repeat 1 span region, was frequently observed in Iranian SARS-CoV-2 samples (16).

In Iran, various variants analogous to those observed in other regions emerged during the onset of the corona disease. These include alpha (B.1.17 and Q.1-Q.8), beta (B.1.351, B.1.351.2 and B.1.351.3), and gamma (P.1, P.1.1, P.1.2). 17.2, AY.3, AY.2 and AY.1), gamma (P.1, P.1.1, P.1.2), epsilon (B.1.427, B.1.429), Eta (B.1.525), Uta (B.1.526), Omicron (BA.2), Kappa (B.1.617.1), 1.617.3, Mu (B.1.621, B.1.621.1), Zeta (P.2) and Lambda (C.37) (17, 18). Among these variants, the alpha, beta, delta and gamma variants appeared in several waves of the disease and had the ability to transmit and cause disease, and it was necessary to use a vaccine against them (17, 18).Variations in the SARS-CoV-2 genome are natural and can interfere with the adaptation between host and virus. The mutations in the Delta and Omicron variants, for instance, have been shown to enhance viral fitness, thereby increasing replication and transmissibility (19).Consequently, further investigation into these mutations is essential for the development of effective prevention, control, and preparedness programs, along with suitable vaccines. A total of 161 nonsynonymous variations were identified along the entire coding S gene, with A262T, D614G and P863H exhibiting a high frequency. These variations were found within HVR1, HVR2 and HVR3, respectively. The present study revealed that the predominant variants (alpha variants) and mutations were in parallel with the evolution of the virus and its fitness.

Acknowledgment

It must be noted that acknowledgments are not applicable in the given context.

Authors' Contribution

HK designed the study. HK and AML provided the materials and equipment. ShS collected samples and performed the study. MMR supervised and analyzed the subjects. MMK, FK, and MM wrote the first draft. MMK GH K assisted in bioinformatics analysis. FK submitted the sequences. All authors read and approved the final manuscript.

Ethics

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of Interest

None

Grant Support

None

Data Availability

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

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