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Research Paper

Comparison of S2 Subunits of the Spike (S) Glycoprotein From Different Strains of SARS-Cov-2 (COVID-19), Aiming to Understand the S2 Role in Virus Transfection Which May Help Its Harness

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ABSTRACT

Introduction: In late 2019, an acute respiratory disease known as SARS-CoV-2 (COVID-19), caused by a novel coronavirus, emerged in Wuhan, China. This disease spread rapidly across China and globally. Many countries were compelled to develop and manufacture vaccines, antigens, diagnostic kits, and antiviral medications to mitigate mortality rates. Severe acute respiratory syndrome coronavirus2 (SARS-CoV2 or COVID-19) uses its spike (S) protein to enable the virus to enter host cells. The viral entry process is linked to the cleavage of the spike (S) protein at the S1| S2 site. This cleavage can take place either at the plasma membrane of the host cell (known as the early pathway) or within the endosomal membrane (referred to as the late pathway), which is determined by the type of host cell involved. Previous research has identified a unique insertion in the S2 region of COVID-19, which may enhance the virus's ability to target cells that express the appropriate proteases and receptors.

Materials & Methods: 3D models of the SARS-CoV and SARS-CoV2 (or COVID-19) spike-proteins (S-protein) were constructed, analyzed, and evaluated using the SARS-CoV spike-structure (PDB No.5X58) as a reference. The structure of CoVs models was reviewed using the online Cn3D V4.3.1 software. Additionally, CoVs sequences were analyzed utilizing the PiTou V3.0.2 software.

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Results: Bioinformatics simulation results indicated that the majority of structural mutations enhancing the efficiency and activity of the S2 subunit were located at the cleavage site (CVs) within the C-terminal region spanning from 654 to 691. Utilizing bioinformatics tools, an analysis of mutations was conducted within the S2 subunit at the excision site and C-terminal region in related CoVs. Additionally, it provided insights into the origin of mutations such as furin and CVs in COVID-19, and compared them with other CoVs.

Conclusion: Most of the mutations that increase the aggressiveness of the S2 subunit were observed in the S2 C-terminal and CVs. Research has shown that furin and some other proteases are involved in processing these mutations. Among these, the transmembrane serine protease 2 (TMPRSS2) is crucial in enabling viral entry through the early pathway.

1. Introduction

1.1. SARS-CoV2 (COVID-19): The appearance and worldwide spread of COVID-19

The SARS-CoV2 virus, which sparked the COVID-19 pandemic, has led to severe respiratory illness and posed a major risk to global health and economies since its discovery in China in late December 2019. Like other coronaviruses, COVID-19 is an enveloped virus that employs its spike (S)-glycoprotein to attach to and penetrate host cells. The spike protein exists as a homotrimer, consisting of three subunits extending from the viral membrane. Understanding the intricate molecular architecture and functionality of this protein is critical to discerning how mutations within it may affect the virus's capacity to infect hosts or escape the immune response [1].

1.2. Spike protein structure: Structural composition of the spike (S)-glycoprotein

The spike protein (S) itself has two domains: the first subunit (S1) domain, which is located outside of the membrane, and the second subunit (S2) domain, which is mainly a transmembrane with a final inside tailing [2]. The S1|S2 units can be broken down into two and five key subdomains, respectively. S1 comprises the NTD (N-terminal domain) as well as the receptor-binding domain (RBD) located at its C-terminal end. In contrast, S2, which engages with the host cell membrane, includes a fusion peptide (FP) subdomain, two heptad-repeat regions (HR1|HR2), a transmembrane domain, and a C-terminal tail.

1.3. Role of S1|S2 subunits: Functional roles of S1|S2 subunits in viral entry

The transmembrane subdomain attaches the S protein to the envelope of COVID-19, while the C-terminal tail resides within the viral particle [3, 4]. The RBD of the S1 protein, which weighs approximately 21 kDa, binds to human ACE2 (human angiotensin-converting enzyme 2) [5]. The virus gains entry into the host cell by fusing with the membrane, using the same receptor targeted by SARS-CoV [5]. After the initial recognition of ACE2 and the virus's attachment, the FP subdomain penetrates the membrane of the host cell [6, 7]. Subsequently, the HR1|HR2 subdomains undergo significant structural shifts, moving towards one another in an antiparallel alignment [8]. When they interact, a six-helix bundle is created, positioning the viral particle close to the host cell membrane to enable membrane fusion and the virus's entry into cell. Importantly, the proteolytic activation of the spike protein has been demonstrated to be a key factor in defining both the host species range and the infectious capacity of coronaviruses [9, 10]. However, most studies have predominantly focused on the immune response, especially how antibodies target the spike and nucleocapsid proteins [10]. The human immune response to COVID-19 can produce antibodies against any of the 29 viral proteins, which include 16 non-structural proteins (NSPs) encoded by the *ORF1a/b* gene [3, 6]. Membrane fusion facilitated by the spike protein requires two separate proteolytic activation stages (PAS) [11, 12].

1.4. Significance of analyzing S2 subunit mutations

Like many other coronaviruses, the S protein of COVID-19 is cleaved at the S2 site by enzymes present in the host., including the serine protease furin [13]. The initial cleavage, known as priming, happens at the S1|S2 junction in certain coronaviruses, while the second necessary cleavage occurs within the S2 region (S2′) [14]. Priming typically readies the S protein for fusion by improving its capacity to bind to receptors or revealing previously con-

cealed cleavage sites (CVs) [8, 15]. The subsequent cleavage induces structural alterations that allow the S-protein to attach to the host-cell-membrane and start the fusion process [16]. Several proteases can execute both the priming and triggering cleavages for coronavirus S-proteins [17, 18]. Although the exact mechanisms of the priming process are not yet fully understood and may differ among viruses, it has been noted that coronaviruses can be activated by proteases either at the plasma membrane or within the endosomal membrane, allowing viral entry through both "early" and "late" pathways [19, 20]. Throughout the maturation of the S-protein, furin or proprotein convertases (PCs) may cleave it [1, 21]. While S2 priming is vital for early pathway entry in MERS-CoV, it doesn't apply to SARS-CoV [7]. Interestingly, MERS-CoV does not require S2 cleavage for entry via the late pathway [4, 22]. SARS-CoV employs the transmembrane serine protease 2 (TMPRSS2) for early pathway entry [2, 9]. However, since the TMPRSS2 expression is confined to epithelial cells, SARS-CoV is able to use endosomal cathepsin L for late pathway entry in cells lacking TMPRSS2 [5]. MERS-CoV employs both TMPRSS2 and cathepsin L for viral entry, and it contains an RSVR sequence at its S2 boundary. This sequence can be cleaved by furin or other proprotein convertases, which are typically found in the secretory pathways of many cell types [17, 21, 22]. The novel polybasic cleavage motif (R-x-x-R) identified at the S1|S2 CVs is likely a result of genetic variation, such as point mutations, insertions, or recombination events, which have led to the insertion of this specific motif [18, 20]. Such sequences are often associated with increased pathogenicity in viruses, as they can enhance furin-mediated cleavage, leading to more efficient processing of viral proteins [11, 14]. This sequence's origin may be elucidated by the selective pressures existing throughout the virus's evolution [8]. Detailed bioinformatics analysis or phylogenetic studies are required to pinpoint the exact origin and evolutionary pathway that led to the emergence of this novel sequence in the viral genome [15]. It is still uncertain whether the novel sequence that includes the polybasic cleavage motif at the S1|S2 site influences furin specificity and enables efficient cleavage by furin [3].

2. Material and Methods

2.1. Predicted structural modeling: 3D structural modeling

Three-dimensional (3D) models of the spike (S) proteins from SARS-CoV and COVID-19 were constructed, analyzed, and assessed based on the SARS-CoV spike protein structure (PDB No.5X58) [10]. To examine the spike protein structures of various coronaviruses (CoVs), the Cn3D V4.3.1 software, which is available online, was utilized [14]. Additionally, coronavirus sequences were submitted to the Prop 1.0 Server [8], and further analyzed using PiTou V3.0.1. The S2 subunit domains of the spike glycoprotein across the coronavirus superfamily (CoVs) were reviewed, about the SARS-CoV structure [gi|2287420714] [11].

Furthermore, the spike protein of the COVID-19 Wuhan-Hu-1 strain was investigated using its GenBank ID (QHD43419.1). Sequences related to the S2 regions of various coronaviruses, including SARS-CoV (AAT74874.1), HCoV-HKU1 (AAT98580.1), Bat-CoV RaTG13 (QHR63300.2), SARS-CoV2 (QHD43416.1), BatCoV-PML (KC869678), Bat-SL-CoVZXC21 (AVP78042.1), Bat-SL-CoV ZC45 (AVP78031.1), BatCoVHKU5 (YP_001039962.1), MERS-CoV (AFS88936.1), Bat-CoV-HKU4 (YP_001039953.1), and Bat-CoV-HKU9 (YP_001039971), were retrieved from GenBank [23], while the S2 sequence of RmYN02 (EPI_ISL_412977) was acquired from GISAID [24]. The sequence alignment for Bat-RmYN02 was performed with the COVID-19 S gene using the GeneiousPrime bioinformatics software, version 2022.1.1 [10].

2.2. Statistical analysis

All statistical analyses were conducted using MEGA V10 and NCBI BLAST online software (tBlastX). Data with multiple groups were analyzed using matched Cn3D V4.3.1 [23, 25, 26], followed by CDC comparisons [16]. Additionally, structures were analyzed using Protein Database Bank (MMDB-PDB) (ID: 6X2A) [27].

3. Results

3.1. Mutation analysis: Identification of key mutations in COVID-19 S2 subunit

Five strains of COVID-19, identified by the World Health Organization (WHO) as VOC (variants of concern), were analyzed to investigate the mutations recognized in the alpha, beta, gamma, delta, and omicron variants. Figure 1 illustrates the key mutations in each COVID-19 strain and their corresponding positions in the S-protein. The mutations in the S2 were assessed through bioinformatics simulations to gain deeper insight into the specificity determinants of these sites. The analysis of mutations indicated that how the S2 subunit affects the infectivity of COVID-19 is diverse, due to the presence of five distinct subdomains within the S2 subunit, each fulfilling unique roles.

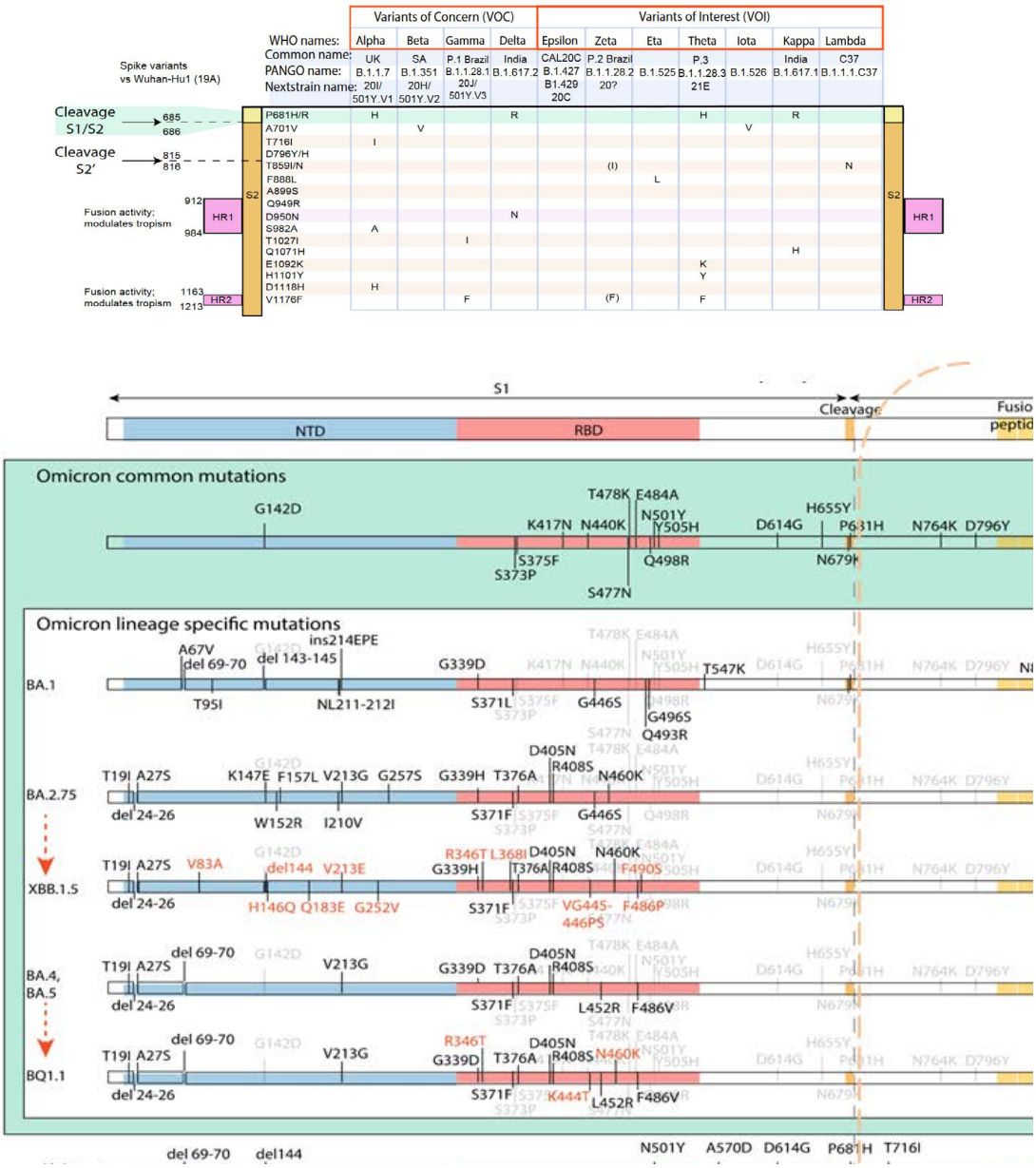


Figure 1. Mutations in SARS-CoV-2 spike protein S2 subunit across WHO-designated VOC

A) Map and table of mutations that have occurred and predicted by WHO for spike protein subunits of all corona strains. The Table Illustrates the key mutations identified in the S2 subunits of the SARS-CoV2 spike protein across five VOC designated by the WHO: Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529). It provides a visual representation of the specific mutations and their precise positions within the S2 subunits of these variants.

B) Mutations occurred in the S2 subunit of the spike protein of the coronavirus in all strains. All of the amino acid changes due to mutations created in the S2 subunit in all Alpha, Beta, Delta, Gamma, Omicron strains have been investigated, this suggests that the difference and mutation in only one amino acid caused the creation of a new strain.

Mutations within the S2 region are common in COVID-19 VOC, with the exception of the Omicron variant, and include changes such as D950N, T716I, D1118H, and S982A (Figure 1).

3.2. Specific mutations in spike-protein

The mutation T716I involves a change from Threonine (T) to Isoleucine (I). Threonine is a polar amino acid that can participate in hydrogen bonding, while Isoleucine is a large hydrophobic amino acid. This substitution might change the conformation of the polypeptide at this po-

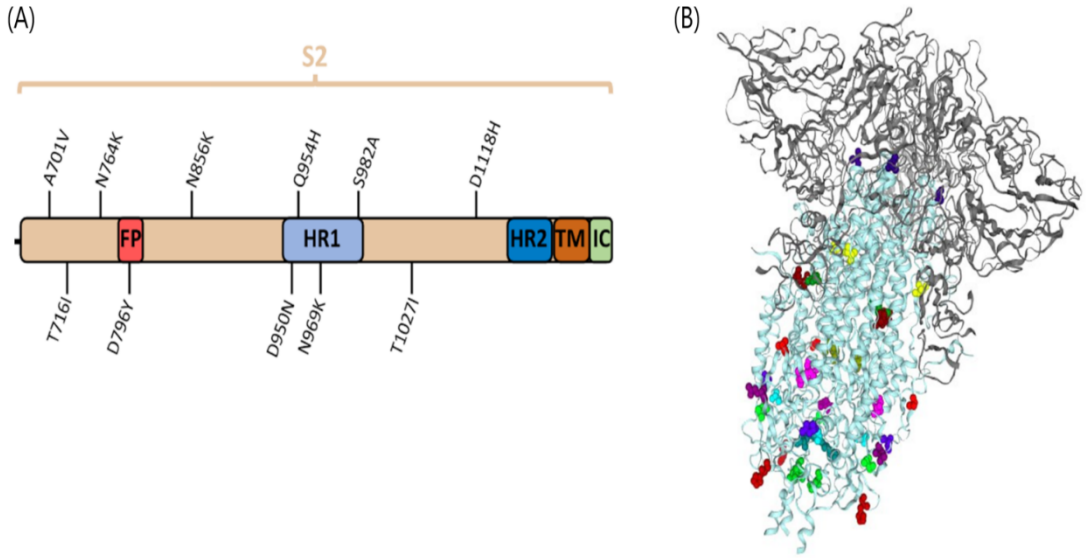


Figure 2. Localization of D950N and S982A mutations in HR1domain of SARS-CoV-2 S2 subunit

A) This bioinformatics prediction figure highlights the strategic positioning of the D950N and S982A mutations within the heptad repeat 1 HR1-domain of the SARS-CoV2 spike protein's S2 subunit. The HR1 domain plays a crucial role in the virus-host membrane fusion process, involving the attachment of the viral membrane to the host cell membrane and their subsequent fusion. The strategic localization of these two mutations within HR1 underscores their potential significance in modulating the fusogenic properties of the spike-protein and consequently impacting viral entry into the host cell and pathogenesis.

B) All mutations occurred on the second subunit(S2) of the coronavirus in strains Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529).

sition. In the D950N mutation, the amino acid aspartic acid (D) is changed to asparagine (N).

Aspartic acid is an acidic amino acid, whereas Asparagine is relatively neutral. This change could potentially create a site for N-glycosylation within the protein structure. The S982A mutation involves a switch from Serine (S) to Alanine (A). Serine is a polar amino acid that can be involved in hydrogen bonding, while Alanine is a small hydrophobic amino acid. This alteration might affect the functional dynamics of the protein at this location. The *D1118H* mutation results in the substitution of Aspartic acid (D) with Histidine (H). Aspartic Acid is an acidic amino acid, while Histidine is a weakly basic amino acid that can easily bind or release protons. This change could modify the protein's charge distribution and possibly impact its function. In the HR1-domain, these two mutations D950N and S982A are found (Figure 2). The Omicron variant, designated as B.1.1.529, carries a considerable number of mutations, particularly in the spike protein's S1|S2 region, which is responsible for the virus's interaction with human cells. A summary of the significant mutations is as follows: N501Y (this mutation is in the RBD and may increase the virus's ability to bind to human cells.), E484A (located in the RBD, this mutation could potentially affect the virus's ability to evade antibodies), K417N (another RBD mutation that might influence the virus's interaction with human cells and immune evasion), T478K (this mutation is also in the RBD and may impact the virus's binding affinity), P681H (located near the FCVs, this mutation might affect the virus's entry into cells), D614G (a mutation found in many variants that may increase transmissibility), H655Y (this mutation is near the FCVs and could influence viral entry into cells), G446S (found in the N-terminal domain (NTD), this mutation might affect antibody recognition), T95I (also in the NTD, this mutation could impact the virus's structure and immune evasion), G142D (located in the NTD, this mutation might alter the virus's ability to evade the immune response), and N679K (this mutation is near the FCVs and may influence the virus's infectivity), Also, the mutations D796Y, N856K, L981F, Q954H, N969K, P1263L, and V1264L were highlighted due to their potential impact on the spike-protein S2 behavior and the overall viral pathogenicity.

3.3. Notable mutations in the S2 of omicron

The Omicron variant of COVID-19 has several mutations across its spike protein, which is divided into S1 and S2 subunits. The S2 subunit is crucial for the vi-

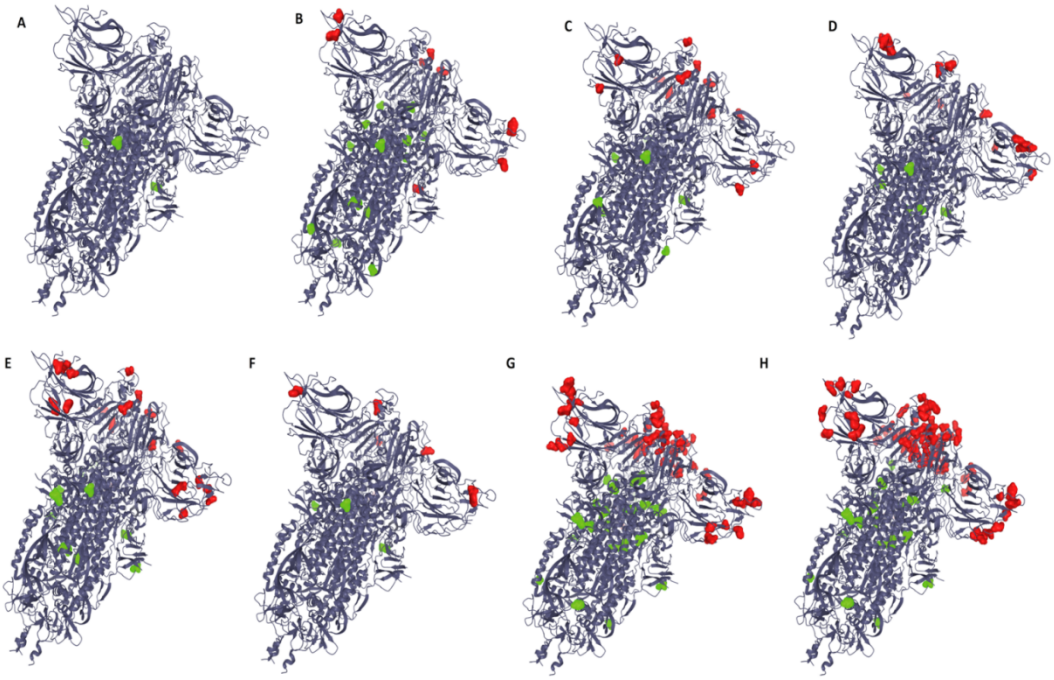


Figure 3. Comparative analysis of S1 and S2 mutations in SARS-CoV-2 variants: structural and functional implications

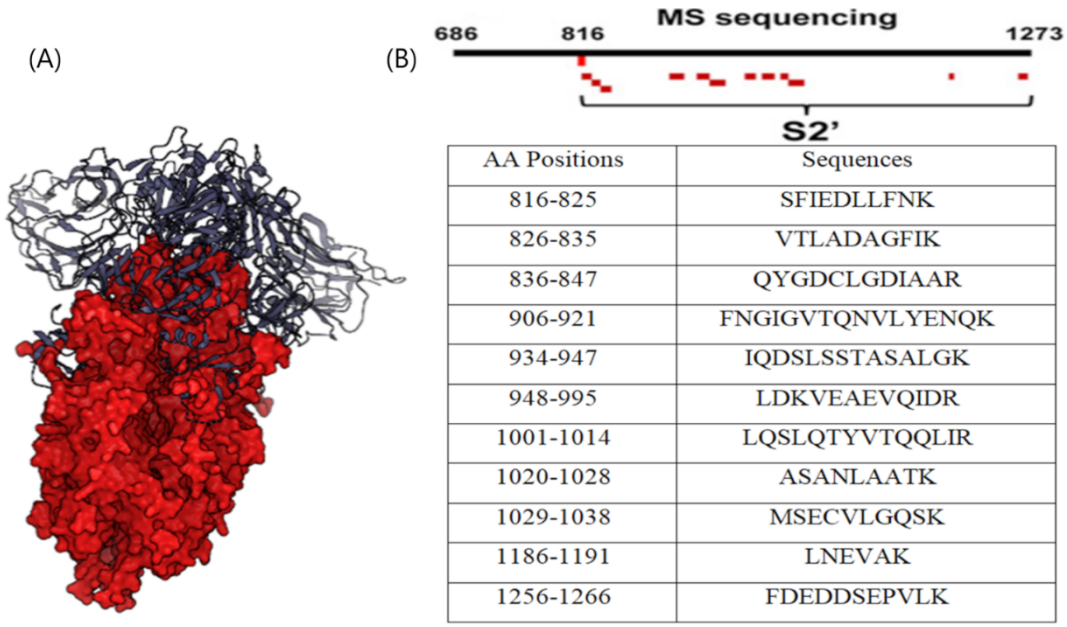
Note: Mutations in WHO-designated VOC and their relative positions in the S-protein: red dots indicate mutations in the S1 subunit, green dots in the S2 subunit.

A) D614G: Enhances spike protein stability, increasing viral load and transmissibility. Induces S2 conformational change, optimizing FP exposure and membrane fusion. Increases ACE2 receptor binding, enhancing S2 fusogenic properties and infectivity; B) Alpha (B.1.1.7): T716I near FP, S982A in HR1-domain, D1118H between HR1 | HR2. May affect viral entry by modulating membrane fusion and S2 stability; C) Beta (B.1.351): A701V near FP, potentially enhancing fusogenic properties by increasing hydrophobicity and membrane-inserting capability; D) Delta (B.1.617.2): D950N in HR1, possibly affecting S2 stability and conformation, disrupting HR1 | HR2 interaction; E) Gamma (P.1.B.1.28): T1027I near C-terminal (HR2 domain), could modulate membrane fusion by altering HR1 | HR2 interplay; F) Epsilon-B1.427&B.1.429: L452R and S13I in S1, indirectly influencing S2 conformation and activity. L452R enhances ACE2 binding, potentially priming S2 for efficient membrane fusion; G) Omicron (B.1.1.529): N764K, D796Y in FP; Q954H, N856K, N969K in HR1. May impact viral entry by modulating membrane fusion, altering S2 stability, and contributing to immune evasion; H) Omicron-XBB.1.5: F486P in RBD increases ACE2 binding affinity. No specific S2 mutations, but enhanced RBD binding may induce conformational changes influencing S2 function, potentially optimizing membrane fusion and viral entry. These mutations across variants affect S-protein stability, receptor binding, membrane fusion, and immune evasion, ultimately impacting viral transmissibility and infectivity.

rus's ability to fuse with the host cell membrane [11]. The identified mutations in the S2 are visible in Figure 3. These studies analyzed the genetic sequence of the Omicron variant and compared it with previous variants to identify mutations that may influence the virus's functionality, such as its fusion capacity, structural stability, and immune evasion [6]. Here are 7 notable mutations in the S2 of the Omicron variant: D796Y (this mutation significantly impacts the virus's neutralization sensitivity, making it more resistant to certain antibodies [10]), N856K (a mutation that reduces the virus's fusion capacity, which is why subsequent Omicron variants lost this mutation to regain fusogenicity [3]), L981F (similar to N856K, this mutation also reduces the fusion capacity of the virus [9]), Q954H (affects the conformation of the S2 subunit and may influence the virus's ability to fuse with host cells [11]), N969K (this mutation could potentially alter the stability of the S2), P1263L (may influence the structural integrity of the S2 [6]), V1264L (could affect the conformation and stability of the S2 [10].

3.4. Structural analysis and implications

The structural analysis of the region between the heptad repeat domains (D1118H) indicated that the residues within this segment facilitate interaction between the heptad repeat domains and the cell membrane, playing a crucial role in repositioning the S2 post-fusion. Moreover, the FCVs is situated within a flexible and disorganized loop on the lateral side of the spike-protein (Figure 3).



AA Positions	Sequences
816-825	SFIEDLLFNK
826-835	VTLADAGFIK
836-847	QYGDCLGDIAAR
906-921	FNGIGVTQNVLYENQK
934-947	IQDSLSSTASALGK
948-995	LDKVEAEVQIDR
1001-1014	LQSLQTYVTQQLIR
1020-1028	ASANLAATK
1029-1038	MSECVLGQSK
1186-1191	LNEVAK
1256-1266	FDEDDSEPVLK

Figure 4. Structural mapping and MS-detected peptides of SARS-CoV-2 S2 subunit: fusion mechanisms insights

A) Depicts the structural representation of the S2 of the SARS-CoV2 spike glycoprotein; B): Maps the SARS-CoV-2 spike peptides detected from MS analysis of the purified S2 fragment

Note: The S2 subunit's peptide coverage is highlighted in red and mapped between amino acid positions 816 and 1266. The figure provides a detailed description of the peptide sequences and their potential roles in membrane fusion, conformational transitions, and stability of the S2. Specifically, the peptide sequences and their potential roles are as follows:

SFIEDLLFNK (816-825): Part of the FP, contains hydrophobic residues (F, L) for membrane insertion and charged residues (D, E) that may contribute to pH-dependent conformational changes, impacting fusogenic properties.

VTLADAGFIK (826-835): Near the FP, may stabilize the FP through hydrophobic interactions (V, L, F, I) and provide flexibility with the G residue, affecting FP conformation and function.

QYGDCLGDIAAR (836-847): Within the HR1-domain, likely engages in hydrophobic packing (Y, L, A), electrostatic interactions (D, R), and disulfide bond formation (C) with HR2 to form the six-helix bundle, critical for membrane fusion.

FNGIGVTQNVLYENQK (906-921): Also, within HR1, may interact with HR2 through hydrophobic packing (F, I, V, L), hydrogen bonding (N, Q), and electrostatic interactions (E, K), affecting HR1-HR2 association and viral infectivity.

IQDSLSSTASALGK (934-947): Located in HR1, likely contributes to monomer interactions and six-helix bundle formation through hydrophobic packing (I, L, A), hydrogen bonding (S, T), and electrostatic interactions (D, K), impacting oligomerization and fusion.

(948-1266): The remaining sequences are described as potentially influencing S2 conformational dynamics, stability, and function through various interactions, including hydrophobic, hydrogen bonding, electrostatic, and disulfide bond formation.

The structural characteristics of the area surrounding the S1|S2 site, which is expected to exhibit flexibility or disorder in the context of COVID-19, may be affected by the overall structure and environment of the entire spike protein in its native configuration. This implies that the conformation and stability of this region, particularly related to the proteolytic CVs (PCVs) between the S1 and S2 subunits, could be impacted by the neighboring structural elements and interactions within the full-length spike protein. This insight underscores the importance of considering the broader structural framework of the spike protein in understanding the functional implications of specific mutations or alterations in this critical region.

3.5. FCVs analysis

The furin-cleavage consensus sequence is present in the S1|S2 region of the COVID-19 spike protein, but it is absent in the MERS-CoV spike protein. The structure surrounding the S1|S2 site in COVID-19, which is predicted to form a flexible and disordered loop, appears to depend on the complete structure of the spike protein (Figure 3). This structural arrangement could af-

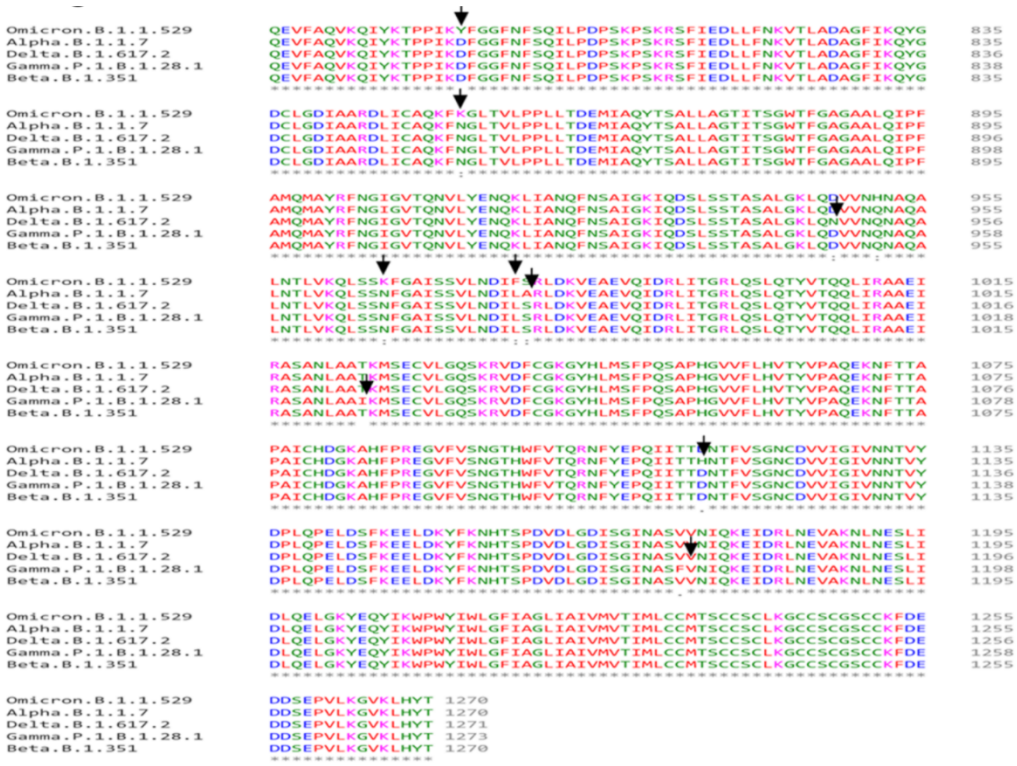


Figure 5. Multiple sequence alignment of SARS-CoV-2 S2 subunit: Variant-specific mutations and conserved regions

Note: This figure presents a comprehensive sequence alignment comparison of the S2 across Omicron (B.1.1.529), Alpha (B.1.1.7), Delta (B.1.617.2), Gamma (P.1.B.1.28.1), and Beta (B.1.351) variants. It highlights the specific amino acid changes in each variant relative to the reference sequence, providing insights into the potential structural and functional implications of these mutations. Notably, the alignment reveals a highly conserved region (residues 1035-1255) encompassing the HR2 domain, TM (transmembrane domain), and a portion of the CT (cytoplasmic tail). This conservation suggests its critical role in the structure and function of the S-protein, making it a potential target for broad-spectrum antiviral therapeutics and vaccine design.

fect the site's accessibility and the manner in which enzymes recognize and process the spike protein (Figure 3). The objective was to investigate the amino acid sequence specificity of the S1|S2 CVs containing the furin motif. The sequences were analyzed according to the 672ASYQTQTNSPRRAR↓SVASQSI692 amino acid sequence in its S2 region (Figure 4).

The novel COVID-19 coronavirus, initially identified in Wuhan, China, in December 2019, exhibited efficient cleavage at this site (Figures 3 and 4).

While the polybasic cleavage motif (R-x-x-R) in the S1|S2 region of MERS-CoV is recognized as a FCVs, its effectiveness in being cleaved by furin was found to be quite limited (Figure 4). The spike (S)-protein sequences of these coronaviruses were examined using the PiTouVer:3.0.2 and ProPVer:1.0.2 prediction tools. The 4 amino acid insertion in the sequence 672ASYQTQTNSPRRAR↓SVASQSI692 of the COVID-19 spike protein, which features the well-established furin cleavage motif (R-x-x-R↓x) marks the S2 CVs, indicated by the arrow (Figure 4). Interestingly, this predicted furin cleavage motif (FCVm) was not present in other viruses within the same clade as COVID-19, such as SARS-CoV-1, the cause of the 2003 epidemic. However, after the COVID-19 sequence was published, it became apparent that a similar polybasic cleavage motif exists in MERS-CoV, the virus responsible for the 2012 outbreak.

3.6. Studying FCVs by bioinformatics modeling

As it is challenging to determine the precise FCVs using bioinformatics modeling alone, additional simulation analysis of the cleaved N-terminal peptides was conducted. Peptides derived from PR↓SVRSV for MERS-CoV, as well as PRR↓ARSV and PR↓RARSV for COVID-19, were detected with lower intensity (the peptides originating from these sequences in MERS-CoV and COVID-19). The lower intensity detection of these peptides suggests that the cleavage at these sites is

less efficient or occurs less frequently compared to other CVs in the respective spike proteins. This finding is important because the efficiency of cleavage at specific sites can affect the virus's ability to enter and infect host cells. This knowledge contributes to our understanding of the factors affecting the infectivity, pathogenicity, and evolutionary relationships of these coronaviruses, potentially guiding the development of targeted interventions to combat current and future outbreaks. However, the cleaved fragments' C-terminal regions consistently started with an ↓SV motif as predicted. This indicates that after cleavage, the resulting protein fragments consistently have their C-terminal regions beginning with the sequence "SV", as was anticipated based on the initial predictions. This finding suggests that the cleavage process is specific and consistent, producing fragments with a predictable sequence pattern. The "↓" symbol indicates the specific site where cleavage occurs, with the "SV" motif following immediately after. These discoveries underscore the involvement of additional elements that contribute to the specificity of PCVs, extending beyond the commonly recognized RxxR motif, which is typically considered the furin cleavage consensus sequence. Bioinformatics modeling has demonstrated that the interaction between the spike protein and the ACE2-receptor triggers the generation of S2 fragments within the target cells, marking a crucial proteolytic event related to spike-mediated membrane fusion. It was discovered that host receptor engagement is vital for proteolytic activation, underscoring the importance of specific residues within the spike-protein.

This highlights a potential targetable mechanism for COVID-19 to infect host cells. For instance, the S982A mutation promotes the "up" state of the RBD by disrupting the interaction with T547, which typically stabilizes the "down" state of the RBD. The terms "up" and "down" refer to the conformational positions of the RBD in relation to the rest of the S-protein structure. In the "down" position, the RBD is situated closer to the core of the S-protein trimer, stabilized by interactions with other regions, such as the threonine residue at position 547 (T547). When the RBD is in the "down" state, it is less accessible to binding to the ACE2-receptor, reducing its ability to facilitate viral entry into host cells. Conversely, in the "up" conformation, the RBD extends away from the core of the S-protein trimer, making it more exposed and available for interaction with the ACE2-receptor on the surface of host cells. This "up" state is believed to be the prefusion conformation that aids the virus in host cells entry.

The alteration in the RBD conformation is partially counteracted by the A570D mutation identified in the Alpha (α) variant of COVID-19. A key difference between the S1|S2 CVs of COVID-19 and MERS-CoV is the presence of three arginine residues situated just upstream of the CVs in COVID-19 (Figure 5). The P3 position, referring to the third amino acid before the CVs, is crucial for how the furin enzyme identifies and processes the protein. Modifications or mutations at the P3 site can alter the efficiency of furin-mediated cleavage, which in turn affects the virus's capacity to infect host cells. Notably, when the P3 residue was mutated from arginine to alanine (R_R_A_R to R_A_A_R), the furin-cleavage efficiency significantly decreased compared to the wild-type sequence of COVID-19 (Figure 5). This P3 residue is a fundamental part of the FCVs, playing a critical role in S-protein activation and the subsequent viral entry into host cells.

3.7. R-x-x-R sequence character

In the COVID-19 spike protein, the FCVs is characterized by the sequence RxxR↓, where R represents arginine, x represents any amino acid, and ↓ indicates the CVs. The arginine (R) residue at the P3 position is essential for the recognition and cleavage by the furin protease.

Mutations at the P3 residue can affect the efficiency of furin cleavage and, consequently, the infectivity and pathogenicity of the virus. This finding underscores the significant impact that a single amino acid substitution can have on protease specificity, which may, in turn, influence the virus's ability to target specific tissues and expand its host range.

3.8. The S1 | S2 cleavage motif phosphorylation

The cleavage motif at the S1|S2 junction in COVID-19 may be phosphorylated by both proline-directed and basophilic kinases. Beyond the FCVs, the insertion of four amino acids in the S1|S2 region introduces new phosphorylation sites positioned adjacent to the main furin motif (Figure 4).

Interestingly, similar phosphorylation sites are also found in the polybasic-proteolyticcleavagesites (PpCVs) of other viral envelope proteins, such as those in H5N1 | H5N8 influenza viruses (Figure 4). It has been observed that mutations in the basic residues at positions +2 and +3 downstream from the CVs at S680, specifically at the C-terminal, affect furin-mediated cleavage (Figures 3 and 4).

Residue 680 within the (S2) region corresponds to the consensus sequence for proline-directed kinases (SP), while residue 686 aligns with the recognition motif for basophilic kinases (RxxS), both belong to two prominent subfamilies of mammalian kinases. The inclusion of four amino acids (PRRA) near the FCVs generates potential phosphorylation targets, particularly for proline-directed kinases at position 680 and basophilic kinases at position 686 (Figure 4).

3.9. The S trimer

In the spike protein trimer, the D1118H mutation results in the formation of a histidine triad composed of three histidine residues in the monomeric form of the protein. This triad aids in the stabilization of the overall trimer structure. Although the exact role of this stabilization is not yet fully understood, it is hypothesized that it may counterbalance local destabilizations caused by mutations like T716I. Additionally, it has been shown that the D570 residue can form an interprotomer hydrogen bond with N856, effectively restoring the bond that maintains the spike protein in its "down" conformation.

The coexistence of opposing mutations within the same variants suggests a balance between preserving spike protein stability and allowing transitions between pre-fusion and post-fusion conformations. The spike (S)-glycoprotein, a large transmembrane protein that coats the viral particle, facilitates the entry of coronaviruses into host cells. Cleavage at the S1|S2 site, particularly at residue R815, is essential for activating the spike protein, a trait observed in COVID-19 variants such as Alpha, Beta, and Delta. In COVID-19, the addition of the PRRARSV motif (with * marking the CVs) at the S1|S2 junction forms a functional polybasic FCVs, a feature that is absent in SARS-CoV and other related coronaviruses.

This structural model emphasizes the S1|S2 cleavage region and the S2 of the spike-glycoprotein across several coronaviruses, including three highly pathogenic human coronaviruses: SARS-CoV (severe acute respiratory syndrome coronavirus), MERS-CoV (Middle East respiratory syndrome coronavirus) and COVID-19, also known as the 2019 novel coronavirus (2019-nCoV).

4. Discussion

4.1. In-depth analysis of COVID-19 S2 subunit

This study presents an in-depth analysis of the S2 of the COVID-19 spike (S) glycoprotein, juxtaposing it with analogous proteins in other coronaviruses (CoVs) to identify critical structural and mutational variations that could significantly inform the design of recombinant vaccine candidates [13]. The findings from this analysis have substantial implications for our understanding of viral entry mechanisms and the development of interventions to mitigate the pathogenicity of COVID-19 [10].

4.2. Mutational landscape and structural dynamics

The mutational landscape and structural dynamics of the S2 revealed significant mutations, particularly at the S1|S2 CVs and the C-terminal region [3]. Mutations such as S982A, D950N, T716I, and D1118H induce conformational changes that critically affect the spike protein's ability to mediate membrane fusion and viral entry [15]. In comparison, the Omicron variant, with mutations like D796Y, N856K, L981F, Q954H, N969K, P1263L, and V1264L, demonstrates enhanced fusogenicity and immune evasion potential [16]. This adaptive capacity underscores the virus's ability to evade host immune responses, consistent with findings by Walls et al. [19] and Hoffmann et al. [13] regarding the critical roles of specific mutations in viral entry mechanisms [13, 19].

4.3. Proteolytic processing and evolutionary adaptation

Bioinformatics studies have revealed that COVID-19 demonstrates enhanced proteolytic processing efficiency at the S1|S2 junction in comparison to other coronaviruses, driven by host proteases such as TMPRSS2 and furin [11]. The distinct presence of a polybasic (multibasic) FCVs, which is absent in closely related viruses like SARS-CoV-1, indicates a possible evolutionary adaptation that has contributed to increased transmission and pathogenicity [14]. Research by Coutard et al. [3] and Hoffmann et al. [13] also highlights the critical involvement of furin-mediated cleavage in the infectivity of COVID-19, reinforcing the importance of targeting these proteolytic pathways in therapeutic interventions [3, 13].

4.4. Advanced 3D structural modeling and computational analysis

Advanced 3D structural modeling and computational analysis have explored the impact of specific mutations on the S2 domain's conformation and function [10]. Mutations like D950N and S982A likely induce structural alterations influencing exposure and functionality

of the FP, thereby impacting the virus's ability to merge with host-cell-membranes [14]. Comparative sequence analysis across a spectrum of coronaviruses, including zoonotic sources like bat coronaviruses, highlights conserved structural motifs and potential antigenic epitopes [10].

These findings offer a framework for pan-coronavirus vaccine design, aligning with studies by Wrapp et al. [7] and Shang et al. [16], which provide foundational insights into spike protein structure and function [7, 16].

4.5. Implications for recombinant vaccine development

The implications for recombinant vaccine development are significant. Despite the high mutation rate, certain regions within the S2 remain highly conserved [14]. Focusing on these conserved regions could result in vaccines conferring broad-spectrum immunity, capable of neutralizing diverse COVID-19 variants [10, 28, 29]. Dynamic vaccine platforms, such as mRNA technologies, which have been demonstrated to be highly adaptable, exemplify a promising approach for incorporating new mutations swiftly, as suggested by Graham et al. (2013) [11]. Given the critical role of host proteases, such as TMPRSS2 and furin, in the viral life cycle, incorporating protease inhibitors into vaccine formulations or using them as adjunct therapies could significantly enhance protective efficacy by blocking crucial steps in viral entry and replication, as supported by studies from Xia et al. and Hoffmann et al. [4, 13].

4.6. Comparative findings and contemporary research

Comparing these findings with contemporary research further underscores their significance. Walls et al. (2020) provided foundational structural insights into the spike protein, particularly the interplay between the S1|S2 subunits in mediating viral entry, which aligns with our observations on the critical role of the S2 in viral fusion and host cell entry [19]. Hoffmann et al. (2020) demonstrated the pivotal role of TMPRSS2 in facilitating COVID-19 entry, emphasizing the relevance of proteolytic processing in the virus's infection mechanism [13]. Coutard et al. (2020) discussed the unique FCVs in COVID-19 and its implications for viral pathogenicity, reinforcing our findings on the enhanced infectivity conferred by furin-mediated cleavage [3].

Shang et al. (2020) examined the structural basis of receptor recognition by COVID-19, highlighting how structural changes in the spike-protein influence host cell binding, supporting our detailed mutational analysis of the S2 and its impact on viral entry mechanisms [16]. Wrapp et al. (2020) focused on the prefusion structure of the s-protein, providing structural insights into potential targets for neutralizing antibodies, which aligns with our emphasis on conserved epitopes within the S2 for vaccine design [7]. Wrobel et al. (2020) provided cryo-EM structures of the spike protein bound to the ACE2 receptor, emphasizing the significance of specific S2 subunit regions in mediating entry and corroborating our findings on the structural importance of the S2 domain in facilitating membrane fusion [22]. Finally, Gui et al. (2017) explored the fusion mechanism of coronaviruses, offering a comparative perspective on the S2 subunit's role across different CoVs and highlighting conserved functional motifs [12]. This supports our analysis of conserved structural elements within the S2 across various Coronaviruses [3]. Hoffmann et al. (2020) analyzed the impact of the D614G mutation on the spike protein's structure and function, revealing that this mutation enhances viral infectivity, this complementing our findings on other critical mutations, such as D950N and S982A, which impact the structural integrity and function of the S2 subunit [13].

Graham et al. (2013) investigated the structural implications of s-protein mutations in emerging variants, emphasizing the need for updated vaccine designs to combat these changes. This supports our call for dynamic vaccine platforms that can rapidly incorporate new mutations in the S2 [11]. Xia et al. (2020) provided detailed insights into the role of the S2 subunit in membrane fusion, highlighting its critical role in the fusion process and reinforcing its importance as a target for therapeutic interventions aimed at blocking viral entry [4]. Suryadevara et al. (2021) examined the neutralizing antibody responses to COVID-19 variants, reinforcing the importance of targeting conserved regions within the S2 for broad-spectrum vaccine efficacy, which aligns with our findings on the potential of conserved epitopes in the S2 [17]. Wang et al. (2021) explored the impact of spike protein mutations on viral fitness and transmission, supporting the critical role of S2 subunit mutations in the virus's adaptive strategies and emphasizing the need to monitor and characterize these mutations for effective vaccine design [1].

Huang et al. (2020) conducted a comprehensive analysis of the spike protein's evolution, providing context for the observed mutational patterns in the S2 and supporting our analysis of evolutionary pressures driving S2 subunit mutations [14]. Yan et al. (2020) focused on the

structural dynamics of the s-protein in different variants, emphasizing the implications of S2 subunit mutations on vaccine efficacy, aligning with our findings on the effect of specific mutations on the S2 subunit's structure and function [9].

4.7. Broader implications and future research

The broader implications of this research underscore the critical role of the S2 in the infectivity and potential for immune evasion of COVID- [17]. The emergence of COVID-19 variants with multiple mutations in the Spike protein has raised concerns regarding their impact on viral-transmissibility, virulence, and immune evasion [11]. In this study, were viewed the mutations in several VOC, including Delta, Gamma, Beta, Alpha, and the recent Omicron variant, along with their reported phenotypes [16]. The Omicron variant (B.1.1.529) of COVID-19 contains numerous mutations in the S-protein, including in the FCVs region (S1|S2) as well as in the S1|S2 subunits [10]. While much attention has been given to the mutations in the S1 and S1|S2 region, the mutations and phenotypic variations in the S2 fragment also need depth stydy [11]. The S2 subunit comprises five subdomains, each with distinct functions, and mutations within this region have been shown to potentially affect COVID-19 infectivity in diverse ways [10]. By integrating advanced structural and computational analyses with comparative virology, this research offers valuable insights for the development of innovative vaccine strategies robust against the evolving viral landscape [10]. Future research should continue to focus on the structural dynamics of emerging variants, pan-coronavirus vaccine development, and host-pathogen interactions to effectively inhibit viral entry and replication [9, 19, 21].

4.8. 3D spike protein analysis by bioinformatics methodology

In this study, a combination of structural modeling, sequence analysis, and statistical analysis was used to investigate the spike-glycoprotein of Coronaviruses [14]. 3D spike protein models of SARS-CoV and SARS-CoV2(COVID-19) were constructed and analyzed based on the SARS-CoV S-structure [10]. The integration of multiple software tools and the inclusion of sequences from various coronaviruses enhance the robustness and credibility of our findings. Bioinformatics simulations were employed to analyze S2 fragment mutants, providing deeper insights into the specificity determinants of these sites.

4.9. Variants and mutations

The research demonstrated that mutations in the S2 of the s-protein have varying impacts on the infectivity of COVID-19, with each of the five subdomains playing unique roles [3]. Our investigation focused on mutations found in five COVID-19 strains labeled as VOC by the WHO: Alpha (α), Beta (β), Gamma (γ), Delta (δ), and Omicron [10]. Mutations in the S2 region are commonly observed across VOCs, except in the most recent Omicron variant. Notable mutations include (S982A), (D950N), (T716I), and (D1118H) [13, 18]. Additionally, our study revealed that the D1118H mutation in the spike trimer facilitates the formation of a histidine triad from three histidine residues within the monomeric S-protein, enhancing the stability of the trimeric structure. The presence of these contrasting mutations within the same variants suggests a regulatory mechanism that balances the structural integrity of the S-protein while enabling necessary shifts between pre-fusion and post-fusion states. Our findings suggest that the S2-subunit is a valuable target for therapeutic development, and additional research is required to thoroughly clarify the factors that regulate proteolytic cleavage at these sites [14].

4.10. Applications for therapeutic interventions

The findings of this study on the mutations and phenotypic changes observed in the Omicron variant, as well as other COVID-19 variants, offer valuable insights for creating more effective therapeutic treatments for COVID-19 [16]. One promising approach is to focus on the S2 of the S-protein, which presents itself as a viable therapeutic target. For example, monoclonal antibodies targeting the S2-subunit have been shown to be effective in neutralizing COVID-19 in both in vitro studies and animal models. Additionally, understanding mutations within the S2 may aid in the design of vaccines tailored to specific variants. This could involve developing mRNA vaccines that incorporate the unique mutations found in the S2 or using adenoviral vectors to deliver the Spike protein with these targeted mutations [6].

4.11. Protease inhibitors platform

To block the action of proteases and preventing the cleavage of the S-protein, protein inhibitors are designed [6]. By inhibiting the proteases, these inhibitors effectively stop the activation of the S2, which is necessary for viral entry. As a result, the virus cannot fuse with the host cell membrane, blocking the infection process. Next-generation vaccines might incorporate protease inhibitors as part of their strategy to prevent infection.

By targeting the S2-mediated fusion step with protease inhibitors, these vaccines can offer an additional layer of protection that extends beyond the usual antibody response to the virus. This approach could help in preventing viral entry and replication even, in cases where the virus manages to evade neutralizing antibodies

4.12. Future research directions

Our analysis of the structural and functional implications of these mutations, especially in the S2-region of the S-protein, indicated that despite the presence of multiple mutations, no structural differences were observed in the new Omicron variant. While this discovery is intriguing, it is crucial to recognize that the effects of these mutations may extend beyond alterations in protein structure alone and could also influence other aspects of viral infectivity and pathogenesis, such as host entry and immune evasion.

Further investigations are required to comprehensively grasp the outcomes of these mutations on the virus and its interactions with the host. The insights gained from this study have far-reaching implications for the development of targeted therapeutic interventions, vaccine design, and public health strategies to combat the ongoing COVID-19 pandemic [10]. By focusing on the S2 and its associated mutations, researchers can leverage advanced drug discovery and vaccine development methodologies to design monoclonal antibodies and mRNA vaccines that effectively combat evolving COVID-19 variants.

Moving forward, it is imperative that the scientific community remains at the forefront of research into the evolving nature of COVID-19 and its variants. Continued investigation into the structural and functional consequences of viral mutations, coupled with the development of novel therapeutic strategies and vaccine platforms, will be crucial in our ongoing battle against this formidable global health threat [7, 30]. Only through a collaborative and multi-disciplinary approach, harnessing the expertise and resources of academia, industry, and public health organizations, we can hope to effectively control the spread of COVID-19 and mitigate the disastrous result of the COVID-19 pandemic on society and global health. By pushing the boundaries of scientific knowledge and innovation, we can work towards a future where we are better prepared to face the challenges posed by emerging infectious diseases and safeguard the health and well-being of populations worldwide. Our study emphasizes and shows that incorporating protease inhibitors into treatment strategies is crucial, as these inhibitors block essential steps the virus needs to enter and replicate within host cells.

Specifically, protease inhibitors disrupt the activation of the S-protein (particularly the S2 subunit), which is a critical step in the fusion of the virus with the host cell. By blocking these steps, protease inhibitors can effectively prevent the virus from infecting new cells and replicating, thus offering a powerful tool in both therapeutic and preventive (e.g. vaccine) strategies against viral infections.

5. Conclusion

The emergence of COVID-19 variants with multiple mutations in the S-Protein has significant implications for viral transmissibility, virulence, and immune evasion. This study focused on the mutations within the S2, which plays a critical role in viral entry and fusion. The analysis revealed that specific mutations, such as D950N and S982A, induce structural changes that impact the virus's ability to infect host cells. Additionally, the study highlighted that while many mutations occur, certain regions within the S2 subunit remain conserved, making them potential targets for broad-spectrum vaccines.

Through advanced 3D modeling and bioinformatics simulations, the study provided insights into how these mutations affect the Spike protein's structure and function. The findings suggest that targeting the S2 subunit with monoclonal antibodies or variant-specific vaccines could be an effective strategy for combating evolving COVID-19 variants. Furthermore, the study underscores the importance of incorporating protease inhibitors into therapeutic approaches to block critical steps in viral entry and replication. Overall, this research offers a comprehensive framework for understanding the role of the S2 in COVID-19 biology and pathogenesis. It emphasizes the need for ongoing surveillance of viral mutations and the development of dynamic vaccine platforms capable of adapting to new variants. These efforts are crucial for mitigating the impact of the COVID-19 pandemic and preparing for future challenges posed by emerging infectious diseases.

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Compliance with ethical guidelines

No animal or human samples were used. All methods were carried out according to the relevant guidelines and regulations.

Data availability

All the data associated with this project is presented in this manuscript.

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Authors' contributions

Conceptualization, supervision, project administration, funding acquisition, review and editing: Khosrow Aghaiypour Kolyani; Methodology, formal analysis, validation, data curation: Meisam Akrami and Khosrow Aghaiypour Kolyani; Investigation and writing the original draft: Meisam Akrami; Resources: Meisam Akrami, Khosrow Aghaiypour Kolyani, Maryam Tajabadi Ebrahimi, Ashraf Mohammadi, and Nakisa Zarrabi Ahrabi.

Conflict of interest

The authors declared no conflict of interest.

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