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Impact of *ACE2* gene variations on COVID-19 pathogenicity in Pakistani patients

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ABSTRACT

Background: COVID-19, caused by the SARS-CoV-2 virus, swiftly disseminated and was declared a pandemic. Variations in the *ACE2* gene can impact the virus's ability to bind to ACE2 receptor, potentially influencing an individual's susceptibility and its association with COVID-19 severity across various populations.

Methods: In total, 200 individuals were sequenced for the *ACE2* gene and potential impact of the found variants on the ACE2 protein was assessed using in-silico tools.

Results: Eight variations in the *ACE2* gene were identified in 27 COVID-19 patients, of which four were missense and four were intronic variants. Three variants had a MAF of < 0.01 (c.251C > T, p.Pro86Leu; 15C > G, p.S5S; and c. 91 A > G, p.Lys31Glu). A missense variant, p.Pro86Leu, C > T, TT genotype, was found in 9 out of 200 individuals with an allele frequency of 0.045 and showed a significant association with COVID-19 (P = 0.003). The heterozygous allele of 15C > G, p.S5S, was found with a frequency of 0.02 (8/400) in eight patients, and its CG genotype showed a significant association with COVID-19 (P = 0.0068). The remaining identified variants were not associated with COVID-19 susceptibility.

Conclusion: The *ACE2* gene sequence in Pakistani individuals exhibited a low frequency of identified variants in COVID-19 patients. Overall, only two variants were associated with susceptibility to the disease, possibly contributing to Pakistan's lower COVID-19 mortality and infection rates. However, individuals carrying the mutant variant experienced more severe symptoms.

1. Introduction

The novel coronavirus emerged from animals to humans, got first noticed in December of 2019 in Wuhan, China (Zhou et al., 2020, Jiatong and Wenjun, 2020) and disseminated worldwide. Due to the rapid global transmission of coronavirus and increased infection rate (Khidri et al., 2022), the WHO announced the novel coronavirus (COVID-19) a pandemic disease (Organization, 2020, Rodriguez-Morales et al., 2020). It infected 687 million individuals globally, and about 6.8 million deaths were recorded worldwide. So far, there have been 659 million recovered patients. Over the last two decades, a family of coronaviruses has caused three epidemics, they manifest similar common symptoms in humans, such as cold, fever, fatigue, and decreased or loss of smell/taste (Wu et al., 2020, Dhama et al., 2020).

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There are several scientific challenges to understanding viral-host interaction and the mechanism by which the virus infects hosts. The clinical presentation, progression, and mortality of COVID-19 have shown variations among individuals belonging to different ethnic groups globally (Kopel et al., 2020, AbuRuz et al., 2022). The SARS CoV 2 (severe acute respiratory syndrome coronavirus 2) utilizes spike protein to attach to ACE2 (angiotensin-converting enzyme 2) receptor and infect human cells. This binding is crucial for the virus to replicate and spread, affecting many organs in addition to the lungs. The *ACE2* catalyzation reaction of angiotensin II to angiotensin-(1–7), and the ACE2/angiotensin-(1–7)/MAS system counteracting the renin-angiotensin system (RAS) effects are crucial in sustaining the pathophysiological balance. The virus's direct effects on inflammation, immune response, and infection, in addition to the disturbance of the ACE2/angiotensin-(1–7)/MAS and RAS equilibrium, can cause multiple organ injury in COVID-19 pathogenesis (Vieira et al., 2021, Ni et al., 2020). The structure analysis showed that the interaction of spike's protein receptor binding domain (RBD) and the *ACE2* peptide domain facilitates the recognition and entrance of SARS-COV-2 into the cells, making it a valuable target for the development of specific vaccines, antibodies, and drugs (Tipnis et al., 2000, Ni et al., 2020).

Epidemiological data indicates that COVID-19 has spread disproportionately across various populations. However, despite its rapid transmission, not all persons who have been in close contact with confirmed COVID-19 cases have contracted the disease, suggesting that distinct genetic variants of *ACE2* may influence susceptibility and severity in different ethnicities and populations (Mueller et al., 2020, Jain et al., 2020, Ghosh et al., 2020). Therefore, this study aimed to investigate the role of ACE2 gene variations on COVID-19 pathogenicity in Pakistani patients.

2. Material and methods

2.1. Ascertainment and clinical evaluation

In this study, 100 individuals tested PCR positive for COVID-19, home quarantine and patients admitted to different COVID-19 emergency wards, the Intensive Care Unit of Liaquat University of Medical and Health Sciences (LUMHS) Hospital, Sindh, Pakistan, were enrolled. After tested of antibodies by using SD Biosensor COVID-19 IgM/IgG plus kit and RT-PCR for COVID-19 one hundred healthy exposed individuals from the general population were also recruited as controls. Informed consent was obtained, and blood samples of infected and control individuals were collected for DNA isolation. The laboratory investigations based tests including CBC-ESR, CRP, D-Dimer, ferritin, LDH and ProBNP, were sent to diagnostic research laboratory Hyderabad. The study got approval from the Ethical Review Committee of LUMHS. The SARS-CoV-2 infection was confirmed using reverse transcription polymerase chain reaction (RT-PCR) on a nasopharyngeal swab. The COVID-19 cases were categorized according to the NIH (National Institute of Health) guidelines into mild (COVID-19 symptoms without dyspnea, or abnormal chest imaging, moderate (lower respiratory disease and SpO2 $\geq$ 94%), and severe (SpO2 < 94%, PaO2/FiO2 < 300 mm Hg, respiratory rate > 30 breaths/min, or lung infiltrates > 50%).

2.2. Sequencing and bioinformatics analysis

Genomic DNA was extracted from both COVID-19 patients and controls for *ACE2* gene analysis. The quantification of DNA was performed using a spectrophotometer. Intronic and exonic boundaries were selected for primer design of the *ACE2* (NM_001371415) gene using the Primer 3 bioinformatics tool (Kõressaar et al., 2018). The all exons of *ACE2* gene were amplified by 5 primer pairs as previously described (Sheikh et al., 2014). The amplified products were sequenced through Sanger sequencing method described earlier (Waryah et al., 2013) and human assembly GRCH37/hg19 of *ACE2* (NM_001371415) gene was used for reference alignment.

2.3. Statistical analysis

Among the demographic and clinical variables, categorical data such as gender, symptoms, contact and travel history, COVID-19 severity, presence of COVID-19 antibodies, and D-dimer were shown as frequencies and percentages. The continuous variables, including age, O_2 saturation, erythrocyte sedimentation rate (ESR), ferritin, C-reactive protein (CRP), lactate dehydrogenase (LDH), and pro-brain natriuretic peptide (proBNP) were presented as medians along with their corresponding lowest and highest values. To assess the normal distribution of the continuous data, the Shapiro-Wilk test was used. The Kruskal-Wallis test followed by the Mann-Whitney test was applied to assess the differences between continuous variables based on COVID-19 severity. The genetic data were presented as MAF and genotypes as frequency and/or percentages. To determine the association between genotypes and COVID-19, Fisher's exact test was applied to compare genotype distributions between COVID-19 cases and controls. The IBM SPSS Statistics 20.0 and GraphPad Prism 9.5.0 software were used for Statistical analyses, and a p-value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. Demographics, clinical presentation, and laboratory investigations

Table 1 displays the demographics, laboratory investigations, and clinical presentation of COVID-19 cases including Age, gender, ESR, CRP, Ferritin, D-dimers, ProbPNP, LDH and antibodies. The patients had a median age of 44 years, and the majority were male (64%). Most of the patients (62%) had a severe COVID-19 infection, while 25% had a moderate infection and 13% had a mild infection. All COVID-19-positive individuals were symptomatic and experi-

Table 1
Demographic and clinical profile of patient.

Characteristics		COVID-19 patients
Gender	Male (n; %)	64 (64)
	Female (n; %)	36 (36)
Age (Years)	Median (min–max)	44 (24–76)
Presence of Symptoms	Symptomatic	100 (100)
	Asymptomatic	0 (0)
Contact history	Yes (n; %)	03 (3)
	No (n; %)	97 (97)
Travel history	Yes (n; %)	10 (10)
	No (n; %)	90 (90)
Severity	Mild (n; %)	13 (13)
	Moderate (n; %)	25 (25)
	Severe (n; %)	62 (62)
O_2 saturation (%)	Median (min–max)	89 (66–99)
COVID-19 antibodies	Positive (n; %)	31 (31)
	Negative (n; %)	69 (69)
ESR (mm/1st hour)	Median (min–max)	55 (0–658)
CRP (mg/dl)	Median (min–max)	6.09 (0–133)
Ferritin (ng/ml)	Median (min–max)	650 (12–6151)
LDH (IU/l)	Median (min–max)	603 (75–2456)
Pro-BNP (pg/ml)	Median (min–max)	488 (19–10208)
D-dimer	Positive (n; %)	39 (39)
	Negative (n; %)	61 (61)

COVID: Coronavirus disease; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; LDH: Lactate dehydrogenase; BNP: B-type natriuretic peptide.

enced a range of symptoms, including fever, sore throat, shortness of breath, myalgia, body ache, headache, loss of taste, loss of smell, vomiting, diarrhea, and abdominal pain. The majority of patients had no travel or contact history. COVID-19 antibodies were not detected in most patients (69%). The analyzed biomarkers showed elevated levels above the normal range among the COVID-19 patients. The age, gender, and clinical investigation did not differ ($p > 0.05$), when analyzed according to the COVID-19 severity among patients (Fig. 1).

3.2. Identified ACE2 gene variations and COVID-19 susceptibility

All 18 exons of the *ACE2* gene were sequenced, and eight variations were identified, of which 4 were located in the exonic region and 4 in the intronic region. Out of 100 patients, these variations were found in 27 patients. No variant was detected in healthy controls. In present study the heterozygous allele of c.15C > G, p.Ser5-Ser, was observed with a frequency of 0.02 (8/400) in eight patients, and its CG genotype showed a significant association with COVID-19 (P = 0.0068). The heterozygous allele of c.15C > G was not previously reported in COVID-19 patients, and the 1000 genome database (https://www.internationalgenome.org/) showed a low allele frequency of c.15C > G, p.Ser5Ser, worldwide population (Table 2). Three other missense variations were detected in the *ACE2* peptide domain with low allelic frequency in COVID-19-positive individuals. A novel missense variant, p.Pro86Leu, C > T, TT genotype, was found in 9 out of 200 individuals with an allele frequency of 0.045 and showed a significant association with COVID-19 (P = 0.003) (Table 3). Another novel heterozygous missense variation, c.91 A > G, p.Lys31Glu, was found in five individuals with an allele frequency of 0.013 (5/400) (Table 2), but it did not show any significant association with the disease susceptibility in our population. Moreover, the rare homozygous variant of c777 T > C, p.Ile259Thr, was identified in one individual with an allele

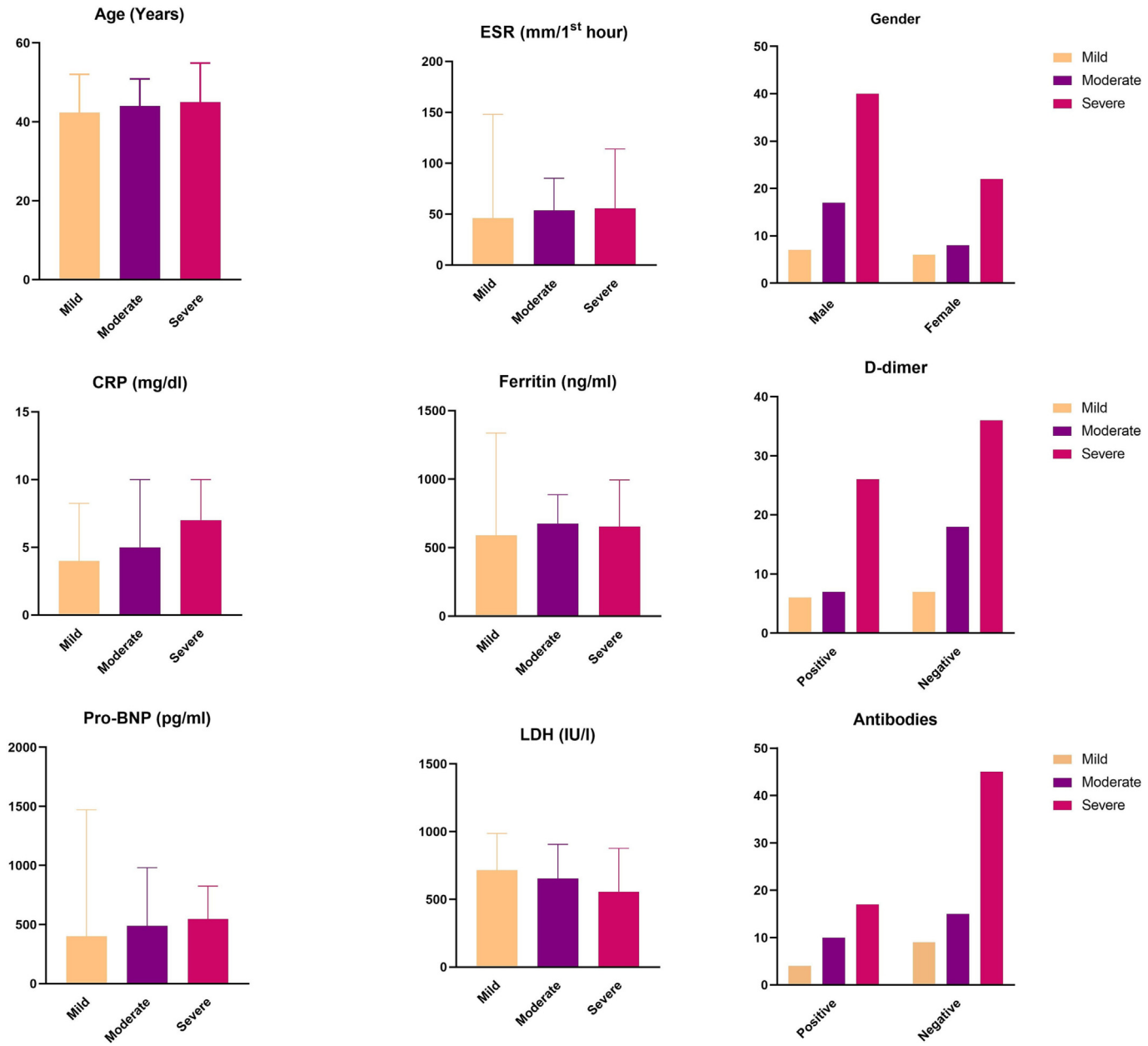

Fig. 1. Distribution of variables according to COVID-19 severity.

Table 2
The minor allele frequency (MAF) of SNPs in exonic and intronic regions of different populations.

Chromosomal location	c.DNA /intronic and Protein location	European (non-Finnish)	Latino	Ashkenazi Jewish	African	East Asian	South Asian	Finnish	Other	Global	In this study n = 200
X:15600897	rs775089013 c.15C > G, p.Ser5Ser	0.000	0.000	0.000	0.000	0.0002793	0.000	0.000	0.000	0.000	0.023 (8/400)
X:15587779	c.777 T > C, p. Ile259Thr	0.000	0.000	0.000	0.0001094	0.000	0.000	0.000	0.000	0.000	0.005 (2/400)
X:15600828	c. 91 A > G, p. Lys31Glu	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.013 (5/400)
X:15594939	c.251C > T, p. Pro86Leu	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.045 (18/400)
X:15587729	rs4646140 c.802 + 24G > A	0.0003950	0.007945	0.000	0.1212 s	0.02503	0.06189	0.0003374	0.01115	NA	0.0025 (1/400)
X:15594812	13025A > G	00	00	00	00	00	00	00	00	00	0.005 (2/400)
X:15582771	2565C > G	00	00	00	00	00	00	00	00	00	0.005 (2/400)
X:15579040	28798A > G	00	00	00	00	00	00	00	00	00	0.005 (2/400)

Table 3
Distribution and association of genotypes of ACE2 variants in patients and controls.

Variants	Genotype	Cases	Controls	P*
c.777 T > C,p.Ile259Thr	TT	99 (99)	100 (100)	1.00
	CC	1 (1)	0 (0)	
c.15C > G, p.Ser5Ser	CC	92 (92)	100 (100)	**0.0068**
	CG	08 (8)	0 (0)	
c.91 A > G, p.Lys31Glu	AA	95 (95)	100 (100)	0.059
	AG	05 (5)	0 (0)	
c.251C > T, p.Pro86Leu	CC	91 (91)	100 (100)	**0.003**
	TT	09 (9)	0 (0)	
rs4646140	GG	99 (99)	100 (100)	1.00
	GA	01 (1)	0 (0)	
13025A > G	AA	99 (99)	100 (100)	1.00
	GG	1 (1)	0 (0)	
2565C > G	CC	99 (99)	100 (100)	1.00
	GG	1 (1)	0 (0)	
28798A > G	AA	99 (99)	100 (100)	1.00
	GG	1 (1)	0 (0)	

P* value is calculated by Fisher exact test.

Table 4
Distribution of SNPs and Genotypes according to severity and gender.

SNP	Genotype	Mild/Moderate Patients	Severe/Critically ill Patients	Gender
c.777 T > C,p.Ile259Thr	CC	0	01	Male
rs775089013 c.15C > G, p.Ser5Ser	CG	04	04	Female: 03 Male: 05
c.91 A > G, p.Lys31Glu	AG	02	03	Female: 03 Male: 02
c.251C > T, p.Pro86Leu	TT	02	07	Female: 05 Male: 04
rs4646140 c.802 + 24G > A	GA	0	01	Female
13025A > G	GG	0	01	Male
2565C > G	GG	0	01	Male
28,798 A > G	GG	0	01	Male
Total	27	08	19	Male: 15 Female: 12

frequency of 0.005 (2/400). Four intronic variants were identified once in four unrelated individuals; the allele rs4646140 c.802 + 24G > A was already reported with low allelic frequencies in different worldwide population (Table 2). Whereas three novel alleles rs900277187 28,798 A > G, 13025A > G and 2565C > G, detected in this study with an allele frequency of 0.0025 (1/400) in our population. the alleles frequency of three novel variants were not reported in the gnomad1000 database (Table 2). These four intronic variants showed no association with disease susceptibility in the study participants (Table 4).

3.3. ACE2 gene variations according to COVID-19 severity

The identified variations were present in 15 males and 12 females, with a ratio of 5:4. Among the 27 patients with identified variations, 19 were severe or critically ill, whereas 8 were mildly or

moderately ill. The majority of the severely or critically ill patients (n = 7) were identified with c.251C > T, p.Pro86Leu variation. The association of genotype with illness and severity has revealed that individuals with mutated genotypes experience severe symptoms and critical conditions. This suggests that genetic changes have a crucial role in determining the severity of illness in these patients (Table 4).

3.4. Functional impact of variations

To predict the functional impact of identified variations on proteins, several bioinformatics tools were used to assess their pathogenicity. Mutation Taster indicated these changes as disease-causing, Provean suggested the change as deleterious, Shift highlighted the change as damaging, and Polyphen2 recommended this change as probably damaging. Hope protein prediction indicated the amino acid variation as pathogenic, and found that the mutated residue is situated in a domain that is crucial for protein activity and has contact with another domain residue. This interaction may be essential for normal protein function, and the mutation may potentially disrupt it, impairing protein function.

The wild-type Ile259 (Fig. 2A) is a conserved residue in the *ACE2* gene and supports the structural integrity of the protein. It is located at the junction between the collectrin-like and the peptidase domain, interacting with surrounding residues and stabilizing the protein structure. Due to the loss of hydrophobicity and external interaction, the mutated Thr259 residue (Fig. 2B) affects regular interaction and normal protein function.

The wild-type lysine residue (Fig. 2A) has been replaced with a mutant glutamic acid residue (Fig. 2D) at position 31. The lysine residue is not located in a conserved domain of the ACE2 gene, but this is essential for the regular activity of the functional protein. The wild-type lysine is positively charged and replaced by a mutant negatively charged glutamic acid residue, which could disrupt this type of interaction and potentially affect the binding of the spike glycoprotein to *ACE2*. A wild-type Pro86 residue (Fig. 2A) is vital for the protein's normal function. The mutated Leu86 residue could potentially disrupt this special conformation and, as a result, interfere with an important interaction between the domains. This disruption could then impact the overall activity of the protein.

The MetaDome bioinformatics tool has suggested that the amino acid substitution at position Lys31 is located in a domain that is intolerant to changes. In contrast, the substitutions at positions Pro86 and Ile259 are located in slightly tolerant domains and are tolerant to changes, respectively (Fig. 3). In predicting the potential impact of genetic variants on protein structure and function, amino acid substitutions that occur in domains that are intolerant to changes are more likely to have a deleterious effect on protein function, while substitutions in domains that are tolerant to changes are more likely to be functionally neutral or have a milder effect.

4. Discussion

COVID-19 has shown disproportionate mortality globally. The mortality rate in the India and South Asia is lower compared to western countries (Jain et al., 2020). Pakistan confirmed 659,116 infections and 14,256 deaths and ranked 31st among countries with a high COVID-19 burden. The mortality rate in Pakistan stands at 2%, which is comparable to India (1.45%) though lower than Iran (4.68%), Italy (3.52%), the UK (3.43%), and other European countries (Burdorf et al., 2020, Sarfaraz et al., 2021). The reasons for this difference in mortality are complex and can be attributed to multiple factors, including age, gender, ethnicity, type of treatment, viral immunogenicity, host genetics, demographics, and sea-

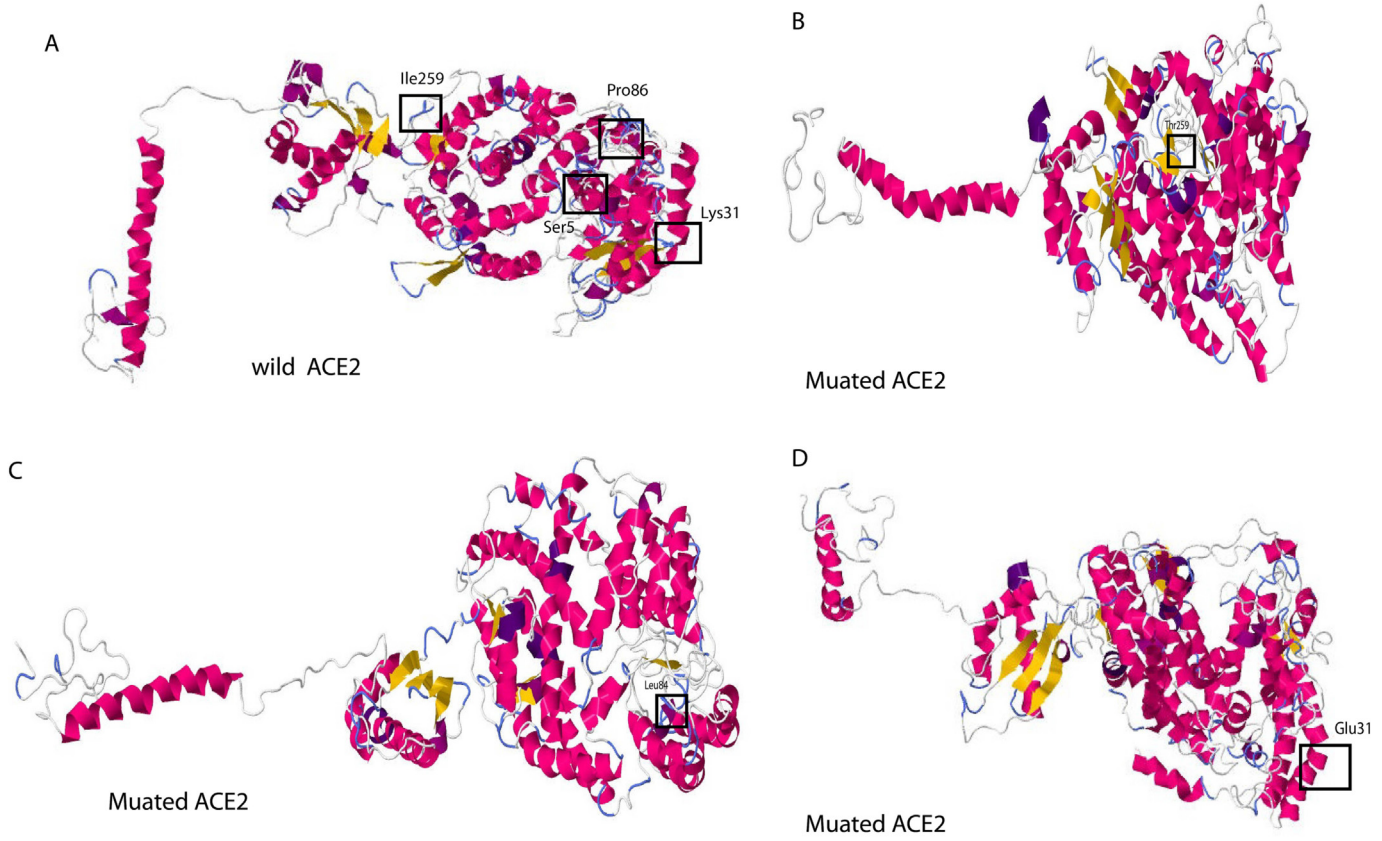

Fig. 2. Phyre2 protein modeling of wild-type ACE2 and Mutant variants.

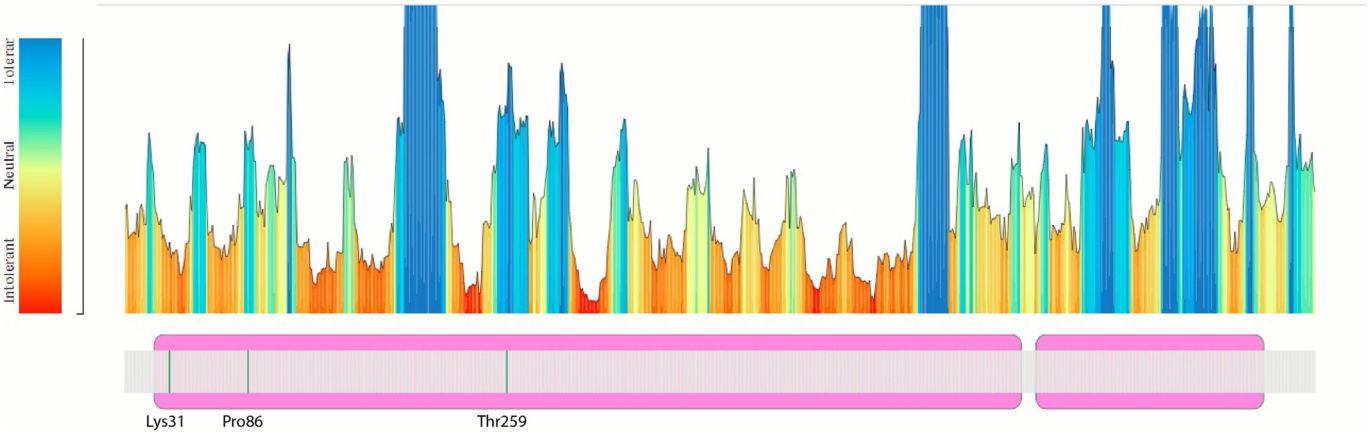

Fig. 3. MetaDome health map showing ACE2 residues Lys31, Pro86, and Thr259.

sonal variations (Abdalla et al., 2022, Banik et al., 2020). Genetic variations due to differences in ethnic and geographical patterns may add to the observed clinical variability of COVID-19; therefore, we investigated whether genetic variations in the *ACE2* gene modulate susceptibility and severity in COVID-19 among Pakistani patients. This is the first study to sequence all exons of the *ACE2* gene in COVID-19 patients and healthy individuals among the Pakistani population (Abdalla et al., 2022).

Genetic variations play a notable role in disease progression and virus-host interaction. The SARS-CoV-2 has a higher binding affinity and binds at least ten times more than SARS-CoV (Cai et al., 2020). The different in silico data suggested that variations within *ACE2* impact dynamic binding and expression levels (Hussain et al., 2020). The structure analysis of SARS-CoV-2 has revealed several key residues (K417, F486, Q498, Y453, T500, Q474, and N501 of the RBD) that are involved in the virus's interaction with the peptide domain of ACE2 and its ability to infect human cells. One of the essential residues is the RBD of the virus's spike protein, which contains several important amino acid residues crucial for ACE2 binding (Hoffmann et al., 2020, Yan et al., 2020).

In the present study, eight variants in the ACE2 gene were identified, of which two were novel missense and three were novel intronic variants. In total, three variants had a minor allele frequency (MAF) of < 0.01 (c.251C > T p.Pro86Leu, c.15C > G, p.Ser5Ser, and c.91A > G, p.Lys31Glu). Two variants, c.251C > T, p.Pro86Leu, and c.15C > G, p.Ser5Ser, were found to be strongly linked with COVID-19, suggesting that they are quite prevalent in our population and may contribute to an increased susceptibility to or severity of COVID-19.

According to the gnomAD database, the c.15C > G, p.Ser5Ser variant is a rare allele with a MAF of < 0.01 in the general population, However, its frequency is slightly higher in East Asian populations, at 0.0002793. The allele c.15C > G, p.Ser5Ser was not previously reported from South Asian population, we have found the heterozygous allele of c.15C > G, p.Ser5Ser, in eight patients with low allele frequency.This shows that genetic variant among individuals could play a role in disease susceptibility and response to treatment. It is necessary to consider these differences in personalized medicine and interpretation of genetic testing. In this study, the c.777 T > C, p.Ile259Thr, was found at an extremely low frequency. The MAF of this variant is reported to be < 0.01 in African populations, indicating its rarity in this group. Bioinformatics tools did not predict any deleterious effects of this amino acid change, which suggests that it is unlikely to significantly impact the protein's structure or function.

Previous studies have indicated the influence of intronic SNPs, such as c.802 + 24G > A (rs4646140), on hypertension and the possibility of interference with the *ACE2* protein product. The rs4646140 variant is not present in the indigenous population, and the MAF ranges from 0 in Indians to 0.13 in Africans, 0.17 in Nigerians (Khayat et al., 2020), and 0.0025 in the Pakistani population, according to the present study. Another three intronic variants (13025A > G, 2565C > G, and 28798A > G) were found with a low MAF (<0.01) in three unrelated severe and critically ill COVID-19 positive individuals, suggesting they may be uncommon in the general population and could be linked to susceptibility and severity to the disease. However, further information is required to establish their significance.

Previous studies have reported that the insertion/deletion (Ins/Del) variant (rs4646994) of the *ACE* gene is linked to higher levels of serum ACE, hypertension, obesity, increased cardiovascular risk, and thrombophilia, all factors that are linked to more severe COVID-19. The deletion/deletion (Del/Del) variant of this gene has also been connected to mortality in acute respiratory distress syndrome and may help explain why COVID-19 is having such a devastating impact in Southern Europe. The frequency of the Del/Del variant differs among populations, with Italy having a much higher representation, especially in older individuals, compared to China. Studies have reported an association between the Del/Del variant and COVID-19-related deaths in 25 European countries (Bellone and Calvisi, 2020, El-Sayed Marei et al., 2023). Other variants, rs4240157 and rs4240157 T > C, have been associated with increased disease severity in some studies, although the underlying mechanism remains under investigation (Mir et al., 2021). The variations rs73635825 and rs143936283 are common in European and African populations (Senapati et al., 2021); however, they were not detected in the Pakistani population in our study. A previous study conducted in Pakistan on the Punjabi ethnicity did not find any link between the only identified p.lys26Arg variant and susceptibility to SARS-CoV-2 (Muhammad et al., 2023), and this variant was not detected in the mixed ethnic population of Pakistan studied in the present research.

The present study confirms that variations in *ACE2*, both previously reported and newly identified, are uncommon in the Pakistani population. The research findings indicate that changes in the SARS-CoV-2 binding area and protein cleavage sites are minimal in COVID-19 positive patients and healthy individuals. The study suggests that two variations in the *ACE2* gene are linked to COVID-19 susceptibility in the Pakistani population, and mutant genotypes may increase severity and mortality risk.

5. Conclusion

In conclusion, the *ACE2* gene sequence in Pakistani individuals exhibited a low frequency of identified variants in COVID-19

patients. Overall, only two variants were found to increase COVID-19 susceptibility among patients, which could explain the lower mortality and infection rates of COVID-19 in Pakistan compared to neighboring and European countries. Nonetheless, patients carrying the mutant variant had more severe symptoms. Further research is required to confirm these findings to understand the role of genetic factors in COVID-19 susceptibility and severity.

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Authors' contributions

All of the authors of the article contributed to the study, including study desig, clinical investigations, laboratory experimentation, analysis of data, and drafting. All of the authors have reviewed the final draft and approved the submission.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Abdalla, M., El-Arabey, A.A., Gai, Z., 2022. Hypertension is still a moving target in the context of COVID-19 and post-acute COVID-19 syndrome. J. Med. Virol.

ABURUZ, S., AL-AZAYZIH, A., ZAINALABDIN, S., BEIRAM, R. & AL HAJJAR, M. 2022. Clinical characteristics and risk factors for mortality among COVID-19 hospitalized patients in UAE: Does ethnic origin have an impact. *PLoS One,* 17**,** e0264547.

Banik, A., Nag, T., Chowdhury, S.R., Chatterjee, R., 2020. Why do COVID-19 fatality rates differ across countries? An explorative cross-country study based on select indicators. Glob. Bus. Rev. 21, 607–625.

Bellone, M., Calvisi, S.L., 2020. ACE polymorphisms and COVID-19-related mortality in Europe. J. Mol. Med. 98, 1505–1509.

Burdorf, A., Porru, F., Rugulies, R., 2020. The COVID-19 (coronavirus) pandemic. Scand. J. Work Environ. Health 46, 229–230.

CAI, Y., ZHANG, J., XIAO, T., PENG, H., STERLING, S. M., WALSH, R. M., JR., RAWSON, S., RITS-VOLLOCH, S. & CHEN, B. 2020. Distinct conformational states of SARS-CoV-2 spike protein. *Science,* 369**,** 1586-1592.

DHAMA, K., KHAN, S., TIWARI, R., SIRCAR, S., BHAT, S., MALIK, Y. S., SINGH, K. P., CHAICUMPA, W., BONILLA-ALDANA, D. K. & RODRIGUEZ-MORALES, A. J. 2020. Coronavirus Disease 2019-COVID-19. *Clin Microbiol Rev,* 33.

EL-SAYED MAREI, Y., ABDALLAH BAYOUMY, A., MOHAMED ABULAZM NASSAR, H., MANSOUR, B. & BAKEIR HAMADY, A. 2023. The Relation between ACE Gene Polymorphism and the Severity of COVID-19 Infection. *International Journal of Microbiology,* 2023**,** 4540287.

Ghosh, D., Bernstein, J.A., Mersha, T.B., 2020. COVID-19 pandemic: The African paradox. J. Glob. Health 10.

HOFFMANN, M., KLEINE-WEBER, H., SCHROEDER, S., KRÜGER, N., HERRLER, T., ERICHSEN, S., SCHIERGENS, T. S., HERRLER, G., WU, N. H., NITSCHE, A., MÜLLER, M. A., DROSTEN, C. & PÖHLMANN, S. 2020. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell,* 181**,** 271-280 e8.

Hussain, M., Jabeen, N., Raza, F., Shabbir, S., Baig, A.A., Amanullah, A., Aziz, B., 2020. Structural variations in human ACE2 may influence its binding with SARS-CoV-2 spike protein. J. Med. Virol. 92, 1580–1586.

Jain, V.K., Iyengar, K., Vaish, A., Vaishya, R., 2020. Differential mortality in COVID-19 patients from India and western countries. Diabetes Metab. Syndr. 14, 1037–1041.

Jiatong, S., Wenjun, L., 2020. Epidemiological characteristics and prevention and control measures of Corona Virus Disease 2019 in children. J. Trop. Med. 20, 153–156.

KHAYAT, A. S., DE ASSUMPÇÃO, P. P., MEIRELES KHAYAT, B. C., THOMAZ ARAÚJO, T. M., BATISTA-GOMES, J. A., IMBIRIBA, L. C., ISHAK, G., DE ASSUMPÇÃO, P. B., MOREIRA, F. C., BURBANO, R. R., RIBEIRO-DOS-SANTOS, A., RIBEIRO-DOS-SANTOS Â, K., DOS SANTOS, N. P. C. & DOS SANTOS, S. E. B. 2020. ACE2 polymorphisms as potential players in COVID-19 outcome. *PLoS One,* 15**,** e0243887.

KHIDRI, F. F., RIAZ, H., BHATTI, U., SHAHANI, K. A., KAMRAN ALI, F., EFFENDI, S., RANI, K. & CHOHAN, M. N. 2022. Physical activity, dietary habits and factors associated with depression among medical students of Sindh, Pakistan, during the COVID-19 pandemic. *Psychology Research and Behavior Management***,** 1311-1323.

Kopel, J., Perisetti, A., Roghani, A., Aziz, M., Gajendran, M., Goyal, H., 2020. Racial and gender-based differences in COVID-19. Front. Public Health 8.

Kõressaar, T., Lepamets, M., Kaplinski, L., Raime, K., Andreson, R., Remm, M., 2018. Primer3_masker: integrating masking of template sequence with primer design software. Bioinformatics 34, 1937–1938.

Mir, M.M., Mir, R., Alghamdi, M.A.A., Alsayed, B.A., Wani, J.I., Alharthi, M.H., Al-Shahrani, A.M., 2021. Strong association of angiotensin converting enzyme-2 gene insertion/deletion polymorphism with susceptibility to SARS-CoV-2, hypertension, coronary artery disease and COVID-19 disease mortality. J. Personalized Med. 11, 1098.

Mueller, A.L., McNamara, M.S., Sinclair, D.A., 2020. Why does COVID-19 disproportionately affect older people? Aging (Albany NY) 12, 9959.

Muhammad, N., Naeemi, H., Azeem, A., Sadaqat, R., Shehzad, U., Siddique, K., Hassan, U., Raza, A., Rashid, M.U., 2023. Genetic analysis of ACE2 peptidase domain in SARS-CoV-2-positive and SARS-CoV-2-negative individuals from Pakistan. Mol. Biol. Rep. 50, 4309–4316.

Ni, W., Yang, X., Yang, D., Bao, J., Li, R., Xiao, Y., Hou, C., Wang, H., Liu, J., Xu, Y., Cao, Z., Gao, Z., 2020. Role of angiotensin-converting enzyme 2 (ACE2) in COVID-19. Crit. Care 24, 422.

ORGANIZATION, W. H. 2020. WHO characterizes COVID-19 as a pandemic [EB/OL].

Rodriguez-Morales, A.J., Bonilla-Aldana, D.K., Balbin-Ramon, G.J., Rabaan, A.A., Sah, R., Paniz-Mondolfi, A., Pagliano, P., Esposito, S., 2020. History is repeating itself: Probable zoonotic spillover as the cause of the 2019 novel Coronavirus Epidemic. Infez. Med. 28, 3–5.

Sarfaraz, S., Shaikh, Q., Saleem, S.G., Rahim, A., Herekar, F.F., Junejo, S., Hussain, A., 2021. Determinants of in-hospital mortality in COVID-19; a prospective cohort study from Pakistan. PLoS One 16, e0251754.

Senapati, S., Banerjee, P., Bhagavatula, S., Kushwaha, P.P., Kumar, S., 2021. Contributions of human ACE2 and TMPRSS2 in determining host–pathogen interaction of COVID-19. J. Genet. 100, 12.

Sheikh, S.A., Waryah, A.M., Narsani, A.K., Shaikh, H., Gilal, I.A., Shah, K., Qasim, M., Memon, A.I., Kewalramani, P., Shaikh, N., 2014. Mutational spectrum of the CYP1B1 gene in Pakistani patients with primary congenital glaucoma: novel variants and genotype-phenotype correlations. Mol. Vis. 20, 991–1001.

Tipnis, S.R., Hooper, N.M., Hyde, R., Karran, E., Christie, G., Turner, A.J., 2000. A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. J. Biol. Chem. 275, 33238–33243.

Vieira, C., Nery, L., Martins, L., Jabour, L., Dias, R., Simoes, E.S.A.C., 2021. Downregulation of membrane-bound angiotensin converting enzyme 2 (ACE2) receptor has a pivotal role in COVID-19 immunopathology. Curr. Drug Targets 22, 254–281.

Waryah, A.M., Narsani, A.K., Sheikh, S.A., Shaikh, H., Shahani, M.Y., 2013. The novel heterozygous Thr377Arg MYOC mutation causes severe Juvenile Open Angle Glaucoma in a large Pakistani family. Gene 528, 356–359.

Wu, J.T., Leung, K., Leung, G.M., 2020. Nowcasting and forecasting the potential domestic and international spread of the 2019-nCoV outbreak originating in Wuhan, China: a modelling study. Lancet 395, 689–697.

Yan, R., Zhang, Y., Li, Y., Xia, L., Guo, Y., Zhou, Q., 2020. Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2. Science 367, 1444–1448.

Zhou, P., Yang, X.L., Wang, X.G., Hu, B., Zhang, L., Zhang, W., Si, H.R., Zhu, Y., Li, B., Huang, C.L., Chen, H.D., Chen, J., Luo, Y., Guo, H., Jiang, R.D., Liu, M.Q., Chen, Y., Shen, X.R., Wang, X., Zheng, X.S., Zhao, K., Chen, Q.J., Deng, F., Liu, L.L., Yan, B., Zhan, F.X., Wang, Y.Y., Xiao, G.F., Shi, Z.L., 2020. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature 579, 270–273.