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ORIGINAL ARTICLE

Genetic analysis of ACE2 peptidase domain in SARS-CoV-2-positive and SARS-CoV-2-negative individuals from Pakistan

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Abstract

Background The outbreak of coronavirus disease 2019 (COVID-19) has emerged as a serious public health emergency of global concern. Angiotensin converting enzyme 2 (*ACE2*) peptidase domain is important for the cellular entry of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Germline variants in *ACE2* peptidase domain may influence the susceptibility for SARS-CoV-2 infection and disease severity in the host population. *ACE2* genetic analysis among Caucasians showed inconclusive results. This is the first Asian study investigating the contribution of *ACE2* germline variants to SARS-CoV-2 infection in Pakistani population.

Methods In total, 442 individuals, including SARS-CoV-2-positive (n = 225) and SARS-CoV-2-negative (n = 217) were screened for germline variants in *ACE2* peptidase domain (exons 2, 3, 9, and 10) using high resolution melting and denaturing high-performance liquid chromatography analyses followed by DNA sequencing of variant fragments. The identified variant was analyzed by *in silico* tools for potential effect on ACE2 protein.

Results A missense variant, p.Lys26Arg, was identified in one SARS-CoV-2-positive (1/225; 0.4%) and three SARS-CoV-2-negative (3/217; 1.4%) individuals. No significant difference in the minor allele frequency of this variant was found among SARS-CoV-2-positive and SARS-CoV-2-negative individuals (1/313; 0.3% versus 3/328; 0.9%; $P = 0.624$), respectively. The SARS-CoV-2-positive patient carrying p.Lys26Arg showed mild COVID-19 disease symptoms. It was predicted as benign variant by *in silico* tool. No variant was detected in ACE2 residues important for binding of SARS-CoV-2 spike protein.

Conclusion The p.Lys26Arg variant may have no association with SARS-CoV-2 susceptibility in Pakistani population. Whole *ACE2* gene screening is warranted to clarify its role in SARS-CoV-2 infection.

Keywords COVID-19 · SARS-CoV-2 · *ACE2* variants · Pakistan

Abbreviations

ACE2	Angiotensin converting enzyme 2.
COVID 19	Coronavirus disease 2019.
DHPLC	Denaturing High-performance Liquid Chromatography.
HRM	High Resolution Melting.
MAF	Minor allele frequency.
PCR	Polymerase chain reaction.
PD domain	Peptidase domain.
RBD	Receptor binding domain.
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2.
WHO	World Health Organization.

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Introduction

The outbreak of coronavirus disease 2019 (COVID-19) has emerged as a serious pandemic and accounted for 617.60 million cases and 6.53 million deaths worldwide, by October 8, 2022 (https://covid19.who.int/). A novel coronavirus-2019 (2019-nCoV), also named as severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) causes this disease [1]. SARS-CoV-2 infection primarily depends on the interaction of its spike protein with the host receptor, angiotensin converting enzyme 2 (ACE2). ACE2 is the cell membrane receptor of SARS-CoV-2 that mediates viral entry into cells [2–4].

The human *ACE2* gene (NM_001371415.1) is on the short arm of X chromosome (Xp22.2). It has 19 exons that encodes a protein of 805 amino acid residues and has two functional domains: N-terminal peptidase domain (residues 19 to 615) and C-terminal collectrin-like domain (residues 616 to 768) [5]. *ACE2* is expressed in epithelial cells of the lung, intestine, kidney, blood vessels [6], and on the oral mucosa [7]. A recent structural analysis showed several key residues (K417, Y453, Q474, F486, Q498, T500, and N501) of the receptor binding motif in the SARS-CoV-2 spike protein receptor-binding domain (RBD) that interact with the ACE2 peptidase domain (residues Q24, D30, H34, Y41, Q42, M82, K353, and R357) [2, 5]. Another study revealed 17 residues of SARS-CoV-2 RBD contact 20 residues of the ACE2 (residues Q24, T27, F28, D30, K31, H34, E35, E37, D38, Y41, Q42, L79, M82, Y83, N330, K353, G354, D355, R357, and R393) that are encoded by *ACE2* exon 2, 3, 9, and 10 [8]. Taken together, these studies showed that certain amino acid residues in the ACE2 peptidase domain are important for the recognition and cellular entry of SARS-CoV-2.

ACE2 receptor genetic variability may affect the virion entry into the host cell and thus the disease severity [9]. *Cao and colleagues* investigated the *ACE2* in public databases and identified 32 coding variants that affect ACE2 protein [10]. The minor allele frequencies (MAF) of *ACE2* variants differ in different populations, suggesting a variable influence [10]. Subsequently, several variants are reported to perturb the ACE2 stabilization, and interfere with interaction with viral spike protein [11]. The epidemiological data reported a disproportional spread of COVID-19 across various populations. It is transmissible in community settings. Various local clusters of COVID-19 are also reported [12]. However, in such clusters, not all individuals in close contact with confirmed COVID-19 patients acquired this infection [12]. This differential pattern of infection suggests distinct *ACE2* genetic variants might influence SARS-CoV-2 susceptibility in different populations [13, 14]. These studies warrant *ACE2* variants screening in original populations to predict the susceptibility and severity to COVID-19. Screening of *ACE2* variants in Caucasian populations have reported controversial results regarding SARS-CoV-2 infection and COVID-19 disease outcomes [11, 15–20]. Since *ACE2* genetic variability is unknown in COVID-19 patients in Asian populations, the present study aimed to investigate germline variants in peptidase domain (*ACE2* exons 2, 3, 9, and 10; amino acids 1 to 115 and 301 to 433) of ACE2 in COVID-19 patients from Pakistan.

Materials and methods

Study subjects

This study includes 442 Pakistani individuals (≥ 18 years) who were tested for SARS-CoV-2 RNA using reverse transcription polymerase chain reaction (RT-PCR) at the Shaukat Khanum Memorial Cancer Hospital and Research Centre (SKM CH&RC), Lahore between June 07, 2020, and June 28, 2020. Of these, 225 SARS-CoV-2-positive and 217 SARS-CoV-2-negative individuals were retrospectively identified. The blood samples from these participants were collected simultaneously with the nasal swabs as per the National Institute of Health, Islamabad guidelines. Demographics and clinical data of all study participants were collected from the electronic medical records of SKM CH&RC. The Institutional Review Board of the SKM CH&RC approved the study (approval number IRB-20-15-A1) and granted the waiver of the written informed consent from study participants.

Molecular analyses

Genomic DNA was extracted from 3 ml to 5 ml of whole blood, as previously described [21]. Comprehensive screening for *ACE2* (NM_001371415.1) exons 2, 3, 9, and 10 (coding for peptidase domain) was performed by High-Resolution Melting (HRM) Analysis using LightCycler 480-II (Roche Diagnostics, Indianapolis, IN, USA) or Denaturing High Performance Liquid Chromatography (DHPLC) analysis using Wave® DNA Fragment Analysis System (Transgenomic Inc., Omaha, NE, USA). Since the *ACE2* is on X chromosome, hemizygous DNA of unknown male samples were mixed 1:1 with wildtype male DNA prior to the amplification, as previously described [22]. The primers were designed using Primer3 software freely available online (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus). Primer sequences, the setup of polymerase chain reaction (PCR), thermal cycling conditions, and the DHPLC running conditions are available upon request. Each sample revealing variants detected by either HRM or

DHPLC analysis was directly sequenced using BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA). The sequencing reactions were purified with BigDye XTerminator Purification Kit (Applied Biosystems, California, USA) and sequenced on an automated 3500 Genetic Analyzer (Applied Biosystems, California, USA) according to the manufacturer's instructions. Bidirectional genomic DNA sequencing was performed to confirm the presence of a variant.

In silico analysis

A missense variant was analyzed for the potential effect on protein function using the default settings of web tool VarCards that annotates the coding variants using more than 60 genomic data sources, including 23 *in silico* algorithms, to comprehensively obtain predictive score and pathogenicity consequences of missense variants [23].

Statistical analysis

The quantitative variables (ages and vital signs) of the study participants were presented as medians/means along with ranges. The categorical variables (gender, travel and contact history, symptoms, comorbidities, radiological findings, disease severity, assessment outcomes, and MAF of c.77 A > G variant) were presented as frequencies and corresponding percentages. The quantitative and categorical variables data were analyzed using the independent t-test and Fisher's exact test, respectively. All statistical test were two-sided, and the groups were considered as statistically different at *P*-value < 0.05. All statistical analyses were performed using IBM SPSS Statistics 20.0 software.

Results

In total, 442 individuals, comprising 225 SARS-CoV-2-positive and 217 SARS-CoV-2-negative, were included in this study. The median age at enrollment was 47.9 years (range 18.2–77.5) for male individuals (n = 243) and 47.0 years (range 18.0–76.0) for female individuals (n = 199). Compared to SARS-CoV-2-negative individuals, SARS-CoV-2-positive individuals were predominantly male ($P = 0.013$), reported a contact history with confirmed or suspected COVID-19 patients ($P = 0.002$), presented with COVID-19 disease symptoms (< 0.0001), had a high body temperature ($P = 0.002$), showed mild to severe COVID-19 disease ($P < 0.0001$), and had a longer duration of hospital stay ($P = 0.013$). The demographic and clinical characteristics of the study participants are presented in Table 1.

ACE2 exons 2, 3, 9, and 10 were screened for genetic variants using HRM and DHPLC, followed by DNA sequencing. No variants were detected in exon 3, 9, and 10 in SARS-CoV-2-positive and SARS-CoV-2-negative individuals. Only one missense variant in exon 2; c.77 A > G (p.Lys26Arg) (rs4646116) was identified in the cohort. Among females, it was identified as a heterozygote in one SARS-CoV-2-positive individual (1/88; 1.1%) and two SARS-CoV-2-negative individuals (2/111; 1.8%). Among males, it was identified as a hemizygous in one SARS-CoV-2-negative individual (1/106; 0.9%) but not among SARS-CoV-2-positive individuals (n = 137; 0%). There was no significant difference ($P = 0.624$) in the MAF of c.77 A > G among SARS-CoV-2-positive and SARS-CoV-2-negative (1/313; 0.3% vs. 3/328; 0.9%) individuals, respectively (Table 2). There was also no difference ($P = 1.0$) in the MAF of c.77 A > G between SARS-CoV-2-positive female and male individuals (1/176; 0.6% vs. 0/137; 0%), respectively (Table 2). It has been reported as a rare variant among South Asians (MAF = 0.13%; 25/19,044 (gnomAD database: https://gnomad.broadinstitute.org/variant/X-15618958-T-C?dataset=gnomad_r2_1 date last accessed: October 10, 2022). It was predicted as benign by all the 21 protein function prediction algorithms integrated in VarCards with a damaging score of 0. It has also been reported as a likely benign variant in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/variation/780203/?oq=rs4646116&m=NM_001371415.1(ACE2):c.77 A%3EG%20(p.Lys26Arg; date last accessed: October 10, 2022). Based on these findings, p.Lys26Arg was classified as likely benign.

The three SARS-CoV-2-negative individuals, including two females and one male harboring the p.Lys26Arg variant, remained asymptomatic for COVID-19 disease. The solitary SARS-CoV-2-positive female carrying the p.Lys26Arg variant was presented with mild COVID-19 disease symptoms of fever, cough, and sore throat.

Discussion

The clinical presentation of COVID-19 disease is highly variable, ranging from asymptomatic infection to severe form of pneumonia and respiratory or multi-organ failure. This is intriguing that genetic variability may influence the susceptibility to SARS-CoV-2 infection and its severity. The relevance of germline *ACE2* variants as a screening target for the risk assessment of SARS-CoV-2 infection is of interest. Most of such studies have investigated the association between *ACE2* variants and SARS-CoV-2 infection or COVID-19 disease severity using bioinformatics approaches while utilizing the frequencies of *ACE2* genetic variants from public databases [10, 14, 24–28]. Only two

Table 1 Demographics and clinical characteristics of the study participants

Characteristics	SARS-Cov-2-positive (N = 225)	SARS-Cov-2-negative (N = 217)	*P*-value
Median age, years (range)	47.9 (18.2–77.5)	47.0 (18.0–76.0)	0.900[a]
Gender, n (%)			
Male	137 (60.9)	106 (48.8)	0.013[b]
Female	88 (39.1)	111 (51.2)	
Travel history, n (%)			
Yes	12 (5.3)	15 (6.9)	0.553[b]
No	201 (89.3)	190 (87.6)	
Contact history, n (%)[c]			
Yes	144 (64.0)	104 (47.9)	0.002[b]
No	78 (34.7)	103 (47.5)	
Clinical characteristics			
Symptoms, n (%)			
Cough	113 (50.2)	50 (23.0)	< 0.0001[b,d]
Myalgia	102 (45.3)	54 (24.9)	
Fever	101 (44.9)	52 (24.0)	
Sore throat	94 (41.8)	49 (22.6)	
Muscle aches	85 (37.8)	48 (22.1)	
Shortness of breath	26 (11.6)	11 (5.1)	
Chills	23 (10.2)	10 (4.6)	
Headache	17 (7.6)	13 (6.0)	
Diarrhea	5 (2.2)	4 (1.8)	
Vomiting	2 (0.9)	1 (0.5)	
Abdominal pain	2 (0.9)	9 (4.1)	
Asymptomatic	61 (27.1)	120 (55.3)	
Comorbidities, n (%)			
None	122 (54.2)	83 (38.2)	0.0003[b,e]
Cancer	84 (37.3)	112 (51.6)	
Diabetes	13 (5.8)	17 (7.8)	
Hypertension	13 (5.8)	19 (8.8)	
Immunocompromised	5 (2.2)	12 (5.5)	
Pneumonia	5 (2.2)	1 (0.5)	
Chronic liver disease	4 (1.8)	2 (0.9)	
Cardiac disease	4 (1.8)	6 (2.8)	
Respiratory illness	3 (1.3)	3 (1.4)	
Chronic pulmonary disease	1 (0.4)	1 (0.5)	
Acute respiratory syndrome	1 (0.4)	1 (0.5)	
Chronic kidney disease	0	3 (1.4)	
Vital signs, mean (range)			
Temperature	36.7 (36–39)	36.4 (35–40)	0.002[a]
Heart rate	110 (37–183)	115 (70–160)	0.979[a]
Respiratory rate	19 (12–26)	23.5 (12–35)	0.599[a]
Systolic BP, mmHg	135 (90–180)	124.5 (76–173)	0.386[a]
Diastolic BP, mmHg	87 (54–120)	82.5 (50–115)	0.063[a]
Oxygen saturation	82 (65–99)	76.5 (53–100)	0.241[a]
Radiological findings, n (%)			
Normal	23 (10.2)	39 (18.0)	0.084[b,f]
Patchy shadowing	15 (6.7)	11 (5.1)	
Consolidation	2 (0.9)	2 (0.9)	
Reticulonodular infiltrates	1 (0.4)	1 (0.5)	
Disease severity, n (%)			
Mild	141 (62.7)	102 (47.0)	< 0.0001[b,g]
Moderate	27 (12.0)	17 (7.8)	
Severe	6 (2.7)	3 (1.4)	

Table 1 (continued)

Characteristics	SARS-Cov-2-positive (N = 225)	SARS-Cov-2-negative (N = 217)	*P*-value
Asymptomatic	47 (20.9)	85 (39.1)	
Assessment outcomes, n (%)			
Referred to beds	147 (65.3)	85 (39.1)	0.013[b,h]
Discharged	48 (21.3)	55 (25.3)	
Referred to EAR	7 (3.1)	13 (6.0)	
Ventilated	3 (1.3)	1 (0.5)	

BP, blood pressure; EAR, emergency assessment room

[a]Independent t-test

[b]Fisher's exact test

[c]Contact with COVID-19 patients as care-giver, health worker, or being patient in healthcare facility

[d]Asymptomatic versus all symptoms

[e]None versus all comorbidities

[f]Normal versus others

[g]Asymptomatic versus disease

[h]Discharged versus others

Table 2 Genotype and allele frequency of *ACE2* c.77 A > G variant in study participants

Gender	Genotypes and alleles	SARS-CoV-2-positive (N = 225) n (%)	SARS-CoV-2-negative (N = 217) n (%)	*P*-value[a]
Females	AA	87 (98.9)	109 (98.2)	1.0
	AG	1 (1.1)	2 (1.8)	
	GG	0	0	
Females	A allele	175 (99.4)	220 (99.1)	1.0[b,c]
	G allele	1 (0.6)	2 (0.9)	
Males[d]	A allele	137 (100)	105 (99.1)	0.436
	G allele	0	1 (0.9)	
Both	A allele	312 (99.7)	325 (99.1)	0.624
	G allele	1 (0.3)	3 (0.9)	

[a]Fisher's exact test

[b]SARS-CoV-2-positive females versus SARS-CoV-2-negative females

[c]SARS-CoV-2-positive females versus SARS-CoV-2-positive males

[d]Given the location of *ACE2* (NM_001371415.1) on X-chromosome, only the allele frequencies are given for the males

European studies from Germany [17] and Spain [29] investigated the contribution of *ACE2* variants to SARS-CoV-2 susceptibility among SARS-CoV-2-positive and SARS-CoV-2-negative individuals. To our knowledge, this is the first Asian study to screen *ACE2* germline variants in the peptidase domain in the Pakistani population.

No germline variant was detected in any of the 20 ACE2 residues, previously inferred for binding of SARS-CoV-2 spike protein. This is consistent with previous reports [11], 16, 18, 20, 29, 30]. Variants in most of these residues are absent or present with a low frequency (MAF < 0.0006) in the general population (gnomAD database). These findings highlight that coding variants in the ACE2 peptidase domain are very rare and may not confer an increased susceptibility or resistance to SARS-CoV-2 infection.

In the present study, only one *ACE2* missense variant c.77 A > G (p.Lys26Arg) was identified with a frequency of 0.44% (1/225) in SARS-CoV-2-positive and 1.38% (3/217) in SARS-CoV-2-negative Pakistani individuals. The allele frequency of c.77 A > G among SARS-CoV-2-positive (MAF = 0.3%) and SARS-CoV-2-negative (MAF = 0.9%) individuals was not significantly different ($P = 0.624$). In consistent with our results, this variant has been reported in a German population with a frequency of 0.34% (1/297) in SARS-CoV-2-positive and 1.18% (3/253) in SARS-CoV-2-negative individuals [17]. Recently, a high prevalence of this variant (10/164; 6.1%) was reported of in SARS-CoV-2-positive patients from Serbia [15]. However, it was not detected in SARS-CoV-2-positive patients from Italy [11, 18], Spain [29], and Russia [20]. This variant has been reported in gnomAD with MAF of 0.388% and 0.131% in the general population and South Asians, respectively. In our study the solitary SARS-CoV-2-positive patient carrying p.Lys26Arg variant showed mild COVID-19 disease symptoms in agreement with previous reports from Serbia [30] and Germany [17]. Collectively, these findings suggest that the p.Lys26Arg variant may neither be associated with increased SARS-CoV-2 infection nor depict a protective effect.

In the current study, p.Lys26Arg was predicted as a benign variant using VarCards *in silico* analysis tool.

Consistently, this variant has been previously classified as a benign variant using various *in silico* analysis tools including SIFT, MUTTASTER, PROVEAN, PolyPhen-2, CADD, and REVEL [24, 31, 32]. Other studies, based on structural modeling and molecular docking simulations suggested that the p.Lys26Arg increased binding affinity of SARS-CoV-2 spike protein to the *ACE2* receptor and showed increased SARS-CoV-2 infection as compared to the wildtype ACE2 [9, 14, 24, 26, 33, 34]. However, this is contradictory to the p.Lys26Arg frequencies in public databases or previous studies on SARS-CoV-2-negative and SARS-CoV-2-positive individuals [17, 29]. Other computational chemistry reports showed that p.Lys26Arg decreased the SARS-CoV-2/ACE2 electrostatic attraction and binding affinity to the host receptor [25, 27, 28]. Further, functional assays and screening of the p.Lys26Arg variant in larger populations of diverse ethnicities and geographical origin may address this discrepancy.

We restricted our analysis to the *ACE2* peptidase domain variants screening, which have been established as RBD contact residues for SARS-CoV-2. Previous studies investigated the few *ACE2* variants [17, 19, 35–37] or screened the complete gene [11, 20, 29, 38–40] and reported conflicting results. No association was found in Spanish [29, 39], Turkish [41], Russian [20], and British [40] populations. In contrast, different *ACE2* variants were identified in Saudi [36], German [17], Italian [35], Spanish [19], and Polish [37] populations, that were linked with genetic susceptibility to SARS-CoV-2 or severity of the COVID-19 disease. However, most of these variants were in the noncoding region of the *ACE2*. Previously, it has been shown that noncoding variants may perturb the *ACE2* gene activity [18]. These reports suggest that *ACE2* variants, particularly in noncoding regions, may have an impact on infection and disease severity of COVID-19 among diverse populations. Complete *ACE2* gene screening of in larger populations would be helpful for the clear understanding of its genetic association with SARS-CoV-2 infection and the course of COVID-19 disease.

Strength of the current study includes the size of study population that was well calculated through Online Sample Size Estimator (http://osse.bii.a-star.edu.sg/calculation1.php), complying by scientific standards for sample size. Another strength is that all study participants were tested by RT-PCR and stratified as SARS-CoV-2-positive or SARS-CoV-2-negative individuals. Whereas, previous studies reported the prevalence of *ACE2* variants in SARS-CoV-2-positive individuals in comparison with healthy controls who were not tested for SARS-CoV-2 [11, 18, 20, 35, 36, 38, 39]. Limitation of this study includes the screening of only partial coding sequence (exons 2, 3, 9, and 10) of ACE2 peptidase domain. Variant screening was performed using HRM and DHPLC assay with variant detection sensitivity < 100%, that may have missed some of the *ACE2* variants. Functional variants in other coding region of *ACE2* may influence COVID-19 disease occurrence and severity in the Pakistani population.

Conclusion

In summary, this is the first study investigating the germline *ACE2* variants in SARS-CoV-2-positive and SARS-CoV-2-negative individuals from Pakistan. No variants were detected in any of the 20 ACE2 residues, previously reported for binding of SARS-CoV-2 spike protein. The only identified missense variant, (p.Lys26Arg) may not be associated with increased susceptibility or protective effect for SARS-CoV-2 infection in the Pakistani population. Screening of the complete *ACE2* gene is warranted to clarify its role in SARS-CoV-2 infection in the Pakistani population.

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

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Institutional Review Board (IRB) of SKM CH&RC (IRB approval number IRB-20-15-A1, date July 24, 2020).

Consent to participate Owing to the retrospective study, the IRB granted the waiver of the written informed consent from study participants.

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