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Original Article

The severity of COVID-19 in hypertensive patients is associated with mirSNPs in the 3′ UTR of *ACE2* that associate with miR-3658: In silico and in vitro studies

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الملخص

أهداف البحث: يستهدف فيروس كورونا سارس-2 مستقبل الإنزيم المحول للمستضد 2، مما يؤدي إلى ارتفاع معدلات الإصابة بالأمراض وزيادة خطر الإصابة بعدوى كوفيد-19 الشديدة والقاتلة لدى الأفراد المصابين بارتفاع ضغط الدم ومرض السكري. هدفت هذه الدراسة إلى تحديد العلاقة بين زيادة الحساسية والشدة من أجل تقييم تأثيرها في مرضى ارتفاع ضغط الدم المصابين بكوفيد-19 باستخدام النماذج المختبرية والمحاكاة بالكمبيوتر.

طريقة البحث: حددنا 80 موقع ربط الجزيئات الصغيرة من ال"رنا" على الإنزيم المحول للمستضد 2 (لـ جزيئات صغيرة من ال"رنا" مختلفة) بالإضافة إلى 30 تعدد أشكال مفرد للنيوكليوتيدات في مواقع ربط الجزيئات الصغيرة من ال"رنا" لـ المنطقة الغير مترجمة بعد توقف ال"رنا" في جين إنزيم المحول للمستضد 2 باستخدام برامج مختلفة عبر الإنترنت والأدوات. من أغسطس 2020 إلى أغسطس 2021، تم جمع ما مجموعه 200 عينة من مسحة الفم / البلعوم الأنفي من ملتان، باكستان. من أجل قياس ال"دنا" المتمم لجينات تحويل المستضد 2 و مير-3658، تم استخدام مضاعفة الحمض النووي باستخدام تفاعل البلمرة المتسلسل الكمي في الوقت الحقيقي بين مرضى كوفيد-19 وارتفاع ضغط الدم وكذلك الأشخاص الأصحاء.

النتائج: من المثير للاهتمام، أن موقع ربط مير-3658 المقابل لـ المنطقة الغير مترجمة بعد ال"رنا" من الإنزيم المحول للمستضد 2 يحتوي على ثلاثة أشكال متعددة الأشكال للنيوكليوتيدات المفردة (رس1457913029؛

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رس290535757؛ رس1423809569) وتسلسل المنطقة الجينومية كان لديها تعدد أشكال النوكليوتيدات المفردة (رس1024225815) مع نفس اختلاف النوكليوتيدات (رس1457913029) مما قد يزيد من شدة كوفيد-19. وبالمثل، تم العثور أيضا على ثلاثة أشكال أخرى متعددة الأشكال للنيوكليوتيدات المفردة (رس1557852115؛ رس770335293؛ رس1024225815) في مواقع الربط الأولى لـ مير-3658. وجدت الدراسة في المختبر أن الإنزيم المحول للمستضد 2 يؤثر التعبير الجيني على مير-3658 في مرضى كوفيد-19 الذين يعانون أيضا من ارتفاع ضغط الدم. في كلتا الحالتين، أوضحت الدراسة أن نموذج المحاكاة بالكمبيوتر يلتقط نفس الآليات البيولوجية مثل تجارب المختبر.

الاستنتاجات: وبالتالي، يمكن أن تكون هذه الأشكال المتعددة للنيوكليوتيدات المفردة هي العلامات الخاصة المحتملة بسبب وضعها في موقع التضفير لجين الإنزيم المحول للمستضد 2.

الكلمات المفتاحية: الإنزيم المحول للمستضد 2؛ جزيئات صغيرة من الرنا؛ المنطقة الغير مترجمة بعد توقف الرنا؛ الجزيئات الصغيرة للأشكال المتعددة للنيوكليوتيدات المفردة؛ ارتفاع ضغط الدم؛ كوفيد -19؛ خطورة

Abstract

The SARS-CoV-2 virus targets the antigen converting enzyme 2 (*ACE2*) receptor, thus resulting in elevated morbidity and an increased risk of severe and fatal COVID-19 infection in individuals with hypertension and diabetes mellitus.

Objectives: This study aimed to identify the association between increased susceptibility and severity in order to evaluate their impact in hypertensive COVID-19 patients using in vitro and in silico models.

Methods: We identified 80 miRNA binding sites on *ACE2* (for different miRNAs) as well as various 30 SNPs in the miRNA binding sites of the 3′ untranslated region (3′ UTR) in the *ACE2* gene using different online software and tools. From August 2020 to August 2021, a total of 200 nasopharyngeal/mouth swabs samples were collected from Multan, Pakistan. In order to quantify the cDNA of *ACE2* and *miR-3658* genes, we used Rotor Gene qRT-PCR on hypertensive patients with COVID-19 as well as healthy controls.

Results: Interestingly, the binding site of miR-3658 corresponding to the 3′ UTR of *ACE2* featured three SNPs (rs1457913029, C>T; rs960535757, A>C, G; rs1423809569, C>T), and its genomic sequence featured a single SNP (rs1024225815, C>T) with the same nucleotide variation (rs1457913029, C>T) which potentially increases the severity of COVID-19. Similarly, three other SNPs (rs1557852115, C>G; rs770335293, A>G; rs1024225815, C>T) were also found on the first binding site positions of miR-3658. Our in vitro study found that *ACE2* gene expression had an effect on miR-3658 in COVID-19 patients who also had hypertension. In both cases, our analysis demonstrated that the in silico model captured the same biological mechanisms as the in vitro system.

Conclusion: The identified SNPs could represent potential informative signatures owing to their position in the splicing site of the *ACE2* gene.

Keywords: 3′ UTR; *ACE2*; COVID-19; Hypertension; miRNAs; Severity

Introduction

Hypertension is a physiologically complex disease that contributes to morbidity and mortality worldwide. The pathogenesis of hypertension is closely associated with dysfunction in the renin-angiotensin-aldosterone system (RAAS).[1,2] It is well established that *ACE2* functions as a negative regulator of RAAS, and its overexpression has already been associated with several diseases such as heart failure, high blood pressure, and diabetes mellitus. In addition, the *ACE2* receptor has also been reported as a functional receptor that is used by SARS-CoV-2 intrusion into host cells.[3,4]

The 3′ untranslated region (3′ UTR) is a section of RNA that is located at the 3′ end of the coding region and is not translated into protein. This region plays a role in regulating the stability and translation of RNA. Recent studies have shown that there may be a relationship between variations in the 3′ UTR of the *ACE2* gene, which encodes the *ACE2* protein, and the susceptibility and severity of COVID-19. The *ACE2* protein is the receptor that SARS-CoV-2 uses to enter human cells. Variations in the 3′ UTR of the *ACE2* gene have been shown to affect the stability and translation of the *ACE2* mRNA, which can in turn affect the amount of *ACE2* protein expressed on the surface of cells.[5,6]

Some studies have suggested that individuals with certain variations in the 3′ UTR of the *ACE2* gene may be more susceptible to severe COVID-19. However, it is important to note that the relationship between the 3′ UTR and the susceptibility and severity of COVID-19 is complex and still being investigated. Other factors, such as age, comorbidities, and genetic variations, in other genes also play a role in determining the susceptibility and severity of COVID-19.[6,7] It is important to note that the relationship between the 3′ UTR and the susceptibility and severity of COVID-19 is still being investigated and more research is needed to fully understand these relationships.

Furthermore, different studies have demonstrated that miRNAs contribute a signature role to the gene expression of COVID-19 in patient tissues[6,8] as they are considered promising biomarkers owing to their potential ability to regulate the pathogenicity of various diseases.[9,10] In a particular infection, miRNAs serve a crucial function in maintaining homeostasis in the body against certain infections. These miRNAs directly interfere with the control mechanisms that regulate DNA replication, gene expression patterns, and protein synthesis. Furthermore, the expression of miRNAs in host cells appears to target viral generations as well as the functions, including expression interference, translation, and even replication.[6,7,10] Anomalies in the expression of miRNAs play a key role in the entry of severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) into cells, ultimately increasing the risk of SARS-COV-2 infection in patients with hypertension.[6,7,10]

As with other coronaviruses, the invasion of SARS-CoV-2 into a host cell is promoted by recognition of the coronavirus spike glycoprotein (S protein) and its binding to the *ACE2* receptor in the host.[11] All variants present with adapted mutations or alterations in the spike protein region[12] that might increase transmissibility or virulency that directly influence the variations and binding capacity of human *ACE2* gene.[13,14] Mutations in the receptor-binding domain (RBD) of spikes are of significant interest because they may have significant impact on viral replication or propagation and host immune response.[13] However, no previous study has investigated genetic variations in *ACE2*-associated miRNA targeting sites, especially in the 3′ UTR which increase or decrease the binding affinity or elevate the probability of infection by SARS-CoV-2 in patients with hypertension and develop more susceptibility and severity of COVID-19.

The aim of this study was to identify functional mirSNPs and their correlations in the 3′ UTR region of the *ACE2* gene, as well as specific miRNAs that were significantly associated with hypertension and COVID-19 patients, using in silico and in vitro investigations. Furthermore, the impact of mutations on COVID-19 susceptibility and severity was investigated.

Materials and Methods

Screening of 3′ UTR regions

Multiple OPEN SOURCE/FREE online software, such as DIANA Tools, miRTarBase, MiCosm, TargetScan, Miranda, miRDB, miRcode, miRecords, miRWalk and miRO (Supplementary Data) have been utilized to identify miRNAs associated to the 3′ UTR region of *ACE2*.[5] Computations and methodologies are outlined below and allowed us to screen the function of miRNAs that bind to the 3′ UTR region of *ACE2*.

DIANA tools

One server (http://diana.imis.athena-innovation.gr/DianaTools/index.php) was used with DIANA-microT with specialized databases to determine the miRNA-mRNA interactions to predict target relationships for in vitro studies. With this tool, positive and negative regions are interlinked with 3′ UTR and coding regions of miRNA, thus allowing them to operate their functions.[15,16]

miRTarBase

There are more than 50,000 interactions of miRNA-targets in miRTarbase that could be used to screen functional miRNAs, mutations, and various gene interactions. Furthermore, this tool and database can also provide gene-related protein analysis based on western blotting or sequence analysis.[17]

MiRanda

The miRanda tool is used for comprehensive assessment, target prediction, specification, and downregulation prediction of miRNA-s.[18]

TargetScan

The computational targets of miRNAs were estimated by TargetScan so as to match their seeding portion at 7 and 8 conserved regions. Furthermore, this software also performs computations and generates estimations based on the context and scores acquired from the mammalian genome. Finally, for specific target locations, target probabilities and prediction rankings are generated for both in silico and in vitro experiments.[19]

miRDB

This is an algorithm that is being employed for the prediction of miRNA targets-functions by analyzing high throughput sequencing data to assess miRNA-target interactions. This provides miRNA data for mammalian species, including mouse, human, rat, chicken, and dog.[20]

miRecords

This is an online database resource for identifying the locations of miRNA targets in animals. This database is divided into two components and nan online tool. The first component verifies and their functions while the second component features miRNA predictions. The most important aspect of this tool is its interconnection with other available online tools.[21]

miRcode

This is an online programming tool that can predict non-coding regions, including the 5′ UTR, 3′ UTR, and coding regions (CDs) of vertebrates. Furthermore miRcode is linked with TargetScan and can also cross-verify its results.[22]

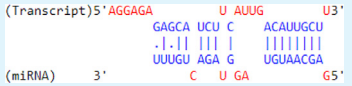
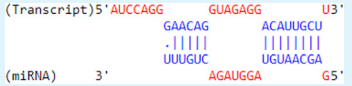
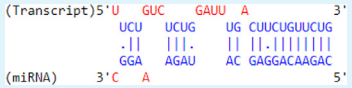
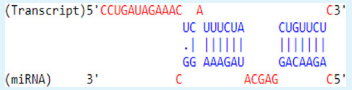
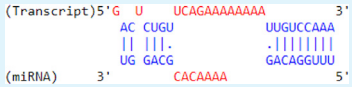
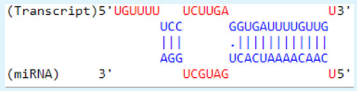
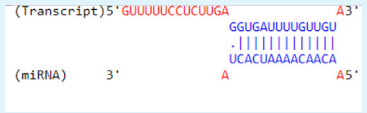
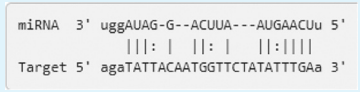
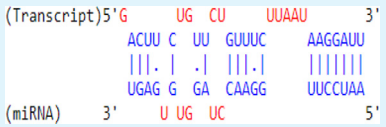
RNA extraction and cDNA synthesis

From August 2020 to August 2021, a total of 200 nasopharyngeal/mouth swabs sample were collected from Multan, Pakistan as approved by the ethical review committee (Regr/Admin/673). In total, samples were taken from 100 COVID-19 patients with a confirmed history of hypertension (stage 02) (Supplementary Data), while 100 samples were collected from healthy controls (no hypertension and COVID-19). Total RNAs were extracted using a commercially available kit (Sigma Aldrich, Trizol; Life Science Technologies) according to the manufacturer's instructions and were stored at temperature of −20 °C. Finally, complementary DNA was synthesized from the extracted total RNA using a QuantiTect Reverse Transcription kit (Qiagen).

qRT-PCR

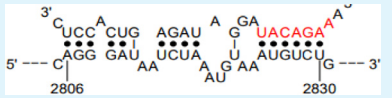
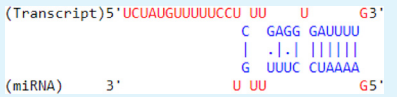
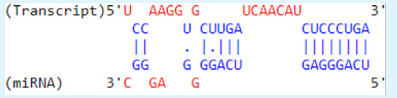
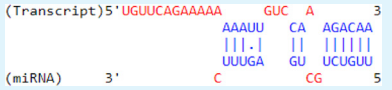
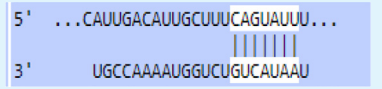
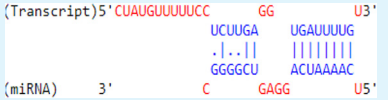
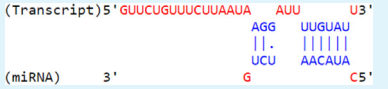
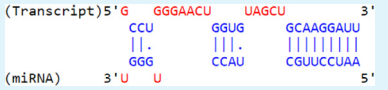
Rotor Gene qRT-PCR (qReal Time PCR) was used to quantify the gene expression levels of ACE-2 and miR-3658 in patients with COVID-19 and hypertension as well as healthy controls. The Mann−Whitney U test and the $2^{-\Delta\Delta Ct}$ method were used to determine relative expression levels.

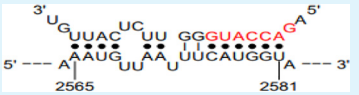
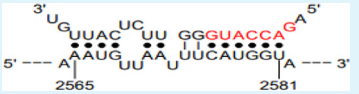
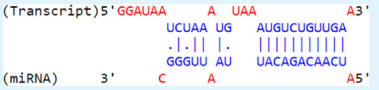
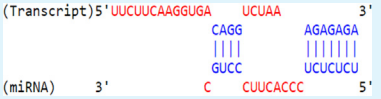
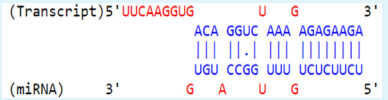
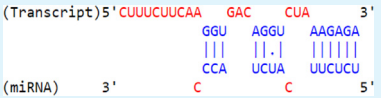
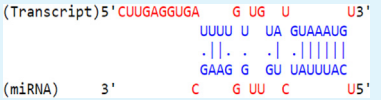
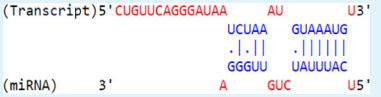
Table 1: List of miRNA target sequences and SNPs at binding sites in the 3′ UTR of *ACE2*.

MicroRNAs	ACE2 Gene Sequences	SNPs	Databases	Screenshots
hsa-miR-3908	AAGCAATG[T/C]CAATGAAGATGC*TC*TCTCCT	rs1436185384T>C	miRDB	
hsa-miR-3908	AAGCAAT[G/A]TCAATGAAGATGC*TC*TCTCCT	rs1479107052G>A	miRDB	
hsa-miR-4773	CAGAACAGA*AGTCAAAT[C/G]C*AGAGACAGA*A	rs1372018909C>G	miRDB STarMirDB	
hsa-miR-4773	GAGAACAGTAGAAATGA*GTTTCT[A/G]TCAGG	rs972411367A>G	miRDB STarMirDB	
Hsa-miR-4520-2-3p	TTT[G/C]GACAA*TTTTTTTTCTGAA*CAGAGTC	rs1294210966G>C	miRDB STarMirDB	
Hsa-miR-3065-5p	ACAACAAAATCACC*TCAAGA[G/C]GAAAAACA	rs1424956426G>C	STarMirDB	
Hsa-miR-3529-3p	TACAA[C/T]AAAATCACC*TCAAGAGGAAAAAC	rs1466778332C>T	miRDB STarMirDB	
Hsa-miR-26b-5p	TTCAAA*TATAG[A/G]AC*CATTGTA*ATATCT	rs1439602685A>G	miRTarBase	
Hsa-miR-362-5p	AATCCTTATTAAGA*AA[C/T]AGAA*CAGA*AGTC	rs1423809569C>T	DianaTool miRDB STarMirDB	
Hsa-miR-7850-5p	CTTT[G/C]GACAA*TTTTTTTTCTGAACAGAGT	rs1294210966G>C	DianaTool miRDB	

(*continued on next page*)

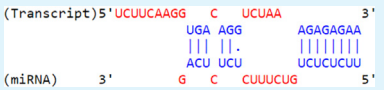
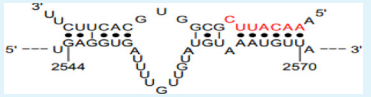
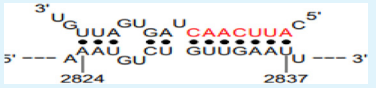
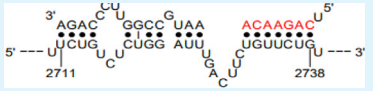
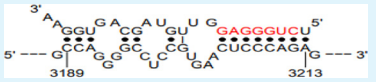
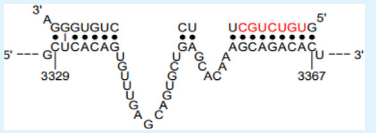
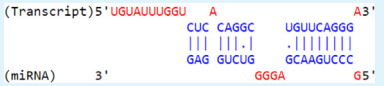
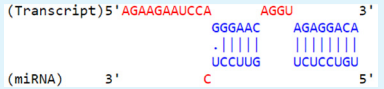
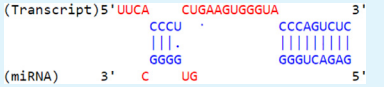
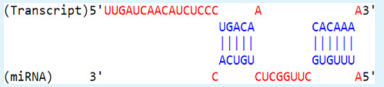
Table 1 (*continued*)

MicroRNAs	ACE2 Gene Sequences	SNPs	Databases	Screenshots
Hsa-miR-144-5p	TGATATCTTTATC[A/G]TCTTATATTTTCTATACC*A*TG	rs1325052635A>G	STarMirDB	
Hsa-miR-641	CAGACATTTA*CATTTAGATT*AT[C/T]CCTG	rs1325998023C>T	STarMirDB	
Hsa-miR-4668-3p	[C/T]AAAATCACC*TC*AAGAGGAAAAACATAGA	rs1466778332C>T	DianaTool	
Hsa-miR-4270	TCAGGGAGATGTTGATCAA*G[C/T]A*CCTTGGA	rs1040962657C>T	DianaTool miRDB	
Hsa-miR-4288	TTGTCTTT[G/C]GACAA*TTTTTTTTCTGAACA	rs1294210966G>C	DianaTool	
Hsa-miR-6755-3p	TGTTGTCTTT[G/C]GACAATTTTTTTTCTGAA	rs1294210966G>C	DianaTool miRDB	
Hsa-miR-429	AAATACTGAAAGCAATG[T/C]CAATG	rs1436185384T>C	TargetScan MirTarBase miRcode	
Hsa-miR-8063	A[C/T]AAAATCACCTCA*A*GA*GGAAAAACATAG	rs1466778332C>T	DianaTool	
Hsa-miR-4666a-3p	AATACAAAATC*CTTATTAAGAAA[C/T]AGAAC	rs1423809569C>T	DianaTool	
Hsa-miR-500b-5p	AATCCTTGCAGCTAC*A[C/G]CAGTTCCCA*GGC	rs1044372223C>G	DianaTool miRDB	

Hsa-miR-591	TACCATGAAAT*T*AACATT[T/C]	rs1387020058T>C	STarMirDB	
Hsa-miR-494	AGAAA[C/T]AGAACA*GA*AG	rs1423809569C>T	STarMirDB	
Hsa-miR-1305	TTTTCAACTTCAGAAATTCA*ACA[G/T]ACATT	rs1245592680G>T	DianaTool STarMirDB	
Hsa-miR-421	TTCAACA[G/T]ACATTTAC*ATTTA*GA*TTATCC	rs1245592680G>T	DianaTool miRDB STarMirDB	
Hsa-miR-7110-3p	TCTCTCTTTAGAC[C/T]TGTCACCTTGAAGAA	rs1178938686C>T	DianaTool	
Hsa-miR-3909	TGTCCTCTACCTGTTCCCTG[G/A]ATTCTTCT	rs1409688114G>A	DianaTool miRDB STarMirDB	
Hsa-miR-942-5p	TCTTCTCTCTTTAGA*C[C/T]TGTCACCTTGAA	rs1178938686C>T	DianaTool STarMirDB	
Hsa-miR-6515-3p	TCTCTTTAGAC*CTGTCA[C/A]CTTGAAGAAAG	rs986013272C>A	DianaTool	
Hsa-miR-4729	ACATTTA[C*/T]ATA*CAA*CA*AAA*TCACCTCAAG	rs1316998365C>T	DianaTool	
Hsa-miR-4729	ACATTTAC*ATTTA*GA*TTAT[C/T]CCTGAACAG	rs1325998023C>T	DianaTool	

(*continued on next page*)

Table 1 (*continued*)

MicroRNAs	ACE2 Gene Sequences	SNPs	Databases	Screenshots
Hsa-miR-6873-3p	TTCTCTCTTTAGAC*[C/T]TGTCACCTTGAAGA	rs1178938686C>T	DianaTool miRDB	
Hsa-miR-543	TAACATTTA*CA*TACAA[C/T]AAAATCACC*TCA	rs1466778332C>T	STarMirDB	
Hsa-miR-4272	AATTCAACA[G/T]ACATTT	rs1245592680G>T	STarmiRDB miRDB	
Hsa-miR-589-3p	ACAGAACAGAAGTCAAAT[C/G]CA*GAGACAGAA	rs1372018909C>G	STarMirDB	
Hsa-miR-3192	CTCTGGGAGTTCAC*GGAGG[C/G]CCCTGGC	rs1182792350C>G	STarMirDB	
Hsa-miR-632	AGTGTCTGCTTTGTGCTCAGCACTGCTCAAACAC[T/C]GTGA*GC	rs1034069205T>C	STarMirDB	
Hsa-miR-345-3p	TCCCTG*A[A/G]CA*GCCTGTGAGACCAAATACA	rs1294683764A>G	DianaTool STarMirDB	
Hsa-miR-6852-3p	TGTCCTCTACCTGTTCCC*TG[G/A]ATTCTTCT	rs1409688114G>A	DianaTool miRDB	
Hsa-miR-4463	GAGACTGGGTA[C/T]CCACTTCAGA*GGGTGAA	rs1013940690C>T	DianaTool	
Hsa-miR-2113	TTT[T/C]GTGTTGTCAGGGAGATGTTGATCAA	rs1363355253T>C	DianaTool STarMirDB	

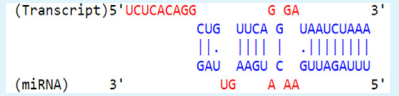
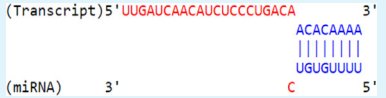
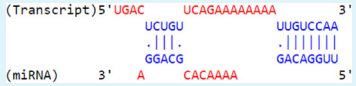
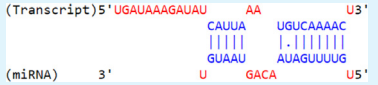
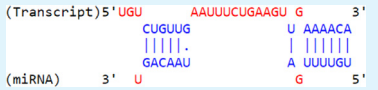
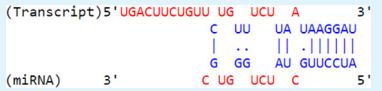
Hsa-miR-200c-3p	TAATACAAAATCCTTATTAAGAAA[C/T]A*GAA	rs1423809569C>T	DianaTool Targetscan miRcode	
Hsa-miR-3658	TTTAAG[A/G]GACTGGGTACCCAC*TTCA*GAGG	rs1198698374A>G	DianaTool	
Hsa-miR-4760-5p	TTTAGATTA*TCCCTGA[A/G]C*AGCCTGTGAGA	rs1294683764A>G	STarMirDB	
Hsa-miR-200b-3p	TAATACAAAATCCTTATTAAGAAA[C/T]AGAA	rs1423809569C>T	DianaTool TargetScan miRcode	
Hsa-miR-5010-3p	TTTT[G/C]TGTTGTCAGGGAGATGTTGATCAA	rs1467544735G>C	DianaTool STarMirDB	
Hsa-miR-4520-3p	TT[G/C]GACAATTTTTTTTCTGAA*CAGA*GTCA	rs1294210966G>C	DianaTool miRDB STarMirDB	
Hsa-miR-2052	AGTTTTGAC*ATTTAA[T/C]GATATCTTTATCA	rs1278818379T>C	DianaTool STarMirDB	
Hsa-miR-2052	TGTTTTCAACTTCAGAAATTC*AACA[G/T]ACA	rs1245592680G>T	DianaTool STarMirDB	
Hsa-miR-502-5p	ATCCTTA*TTAAGAA*A*[C/T]AGAACAGAAGTCA	rs1423809569C>T	DianaTool	

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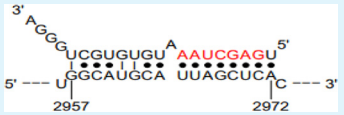
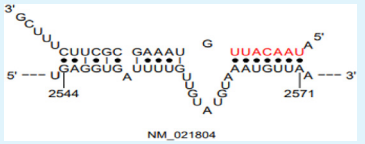
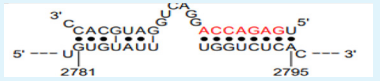
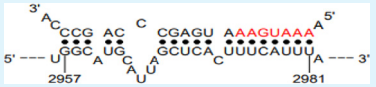
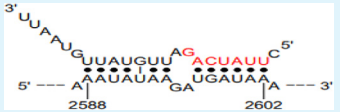
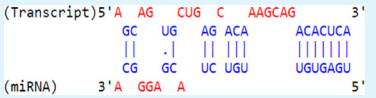
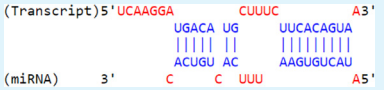
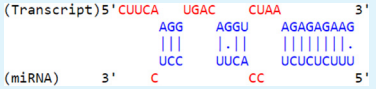
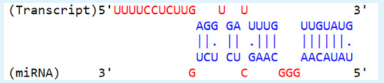
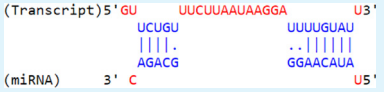
Table 1 (*continued*)

MicroRNAs	ACE2 Gene Sequences	SNPs	Databases	Screenshots
hsa−miR−610	GTG[A/G]GCTAATGC*A*TGCC*A	rs1402794569A>G	STarMirDB	
hsa−miR−3143	TTAACATTTACATACAA[C*/T]AAAAT*CA*CC*TCA	rs1466778332C>T	STarMirDB	
hsa−miR−4786−5p	[G/A]TGAGACCAA*ATA*CACA	rs1342086305G>A	STarMirDB miRDB	
hsa−miR−3646	AATGAAAGTGAGCTAATG[C/T]ATGCCA	rs1362223959C>T	STarMirDB miRDB	
hsa−miR−374a−3p	TTTATC[A/G]TCTTA*TATTT	rs1325052635A>G	STarMirDB	
Hsa-miR-3659	T[G/C]AGTGTCTGCTTTGTGCTCAGCA*CTGCT	rs1481257600G>C	DianaTool	
Hsa-miR-4693-5p	[T/C]TACTGTGAAGAAAGCATGTCATCCTTGA	rs1301618417T>C	miRDB STarMirDB	
Hsa-miR-6833-3p	CTTCTCTCTTTAGACC*TGTCA[C/A]CTTGAAG	rs986013272C>A	DianaTool	
Hsa-miR-500a-5p	C*AATCCTTGCAGCTA*CA*[C*/G]CAGTTCCCAGG	rs1044372223C>G	miRDB	
Hsa-miR-381-3p	C*ATACAA[C/T]AAA*ATCAC*CTCAAGAGGAAAA	rs1466778332C>T	DianaTool	
Hsa-miR-300	AATACAAA*A*TCCTTATTAAGAAA*[C/T]AGAAC	rs1423809569C>T	DianaTool	

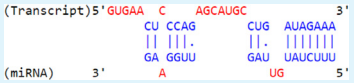
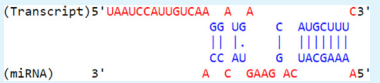
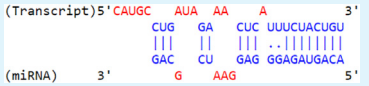
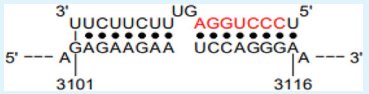
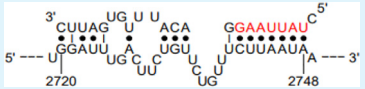
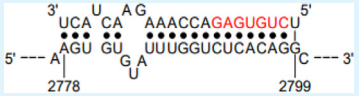
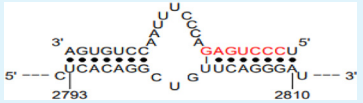
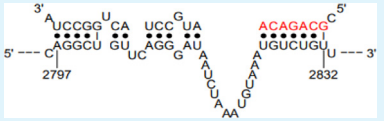
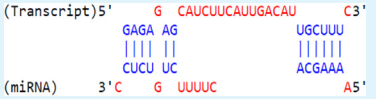
Hsa-miR-7159-3p	TTTCTATC*A[G/A]GCATGCTC*TGGGAGTTCAC	rs1027534608G>A	DianaTool	
Hsa-miR-4422	GAAAGCATGTC*ATC[C/A]TTGACAATGGATTA	rs1329089026C>A	DianaTool	
Hsa-miR-6760-3p	GCACTGTCCTT[A/G]CAAGTGATCCATCCATA	rs1382807063A>G	DianaTool	
Hsa-miR-936	ACAGTAGAAATGAGTT*T*CT[A/G]TCAGGCATG	rs972411367A>G	DianaTool miRDB	
Hsa-miR-4308	TTCCCTG[G/A]ATTCTTCTC*T	rs1409688114G>A	STarMirDB miRDB	
hsa−miR−4477a	TTATTAAGA*AACAGAA[C/T]AGAAGTCAA*ATC*CA	rs1457913029C>T	STarMirDB miRDB	
hsa−miR−4677−3p	GC*CT[G/A]TGAGACCAAATACACAC	rs1342086305G>A	STarMirDB MiRDB	
hsa−miR−125a−5p	ATCCCTGAA*CAGCCT[G/A]TGAG	rs1342086305G>A	STarMirDB MiRCode	
hsa−miR−3166	AA*CA[G/T]ACATTTACATTTAGATTATCCCTGAACA*GCCTG	rs1245592680G>T	STarMirDB	
Hsa-miR-6835-3p	GAAAGCAATG[T/C]CAATGAAGATGCTCTCTC	rs1436185384T>C	DianaTool	

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Table 1 (*continued*)

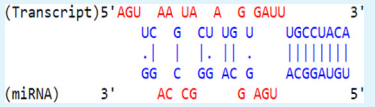
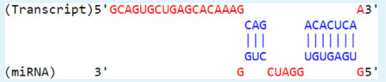
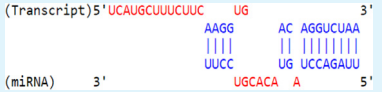
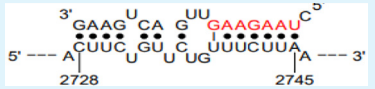
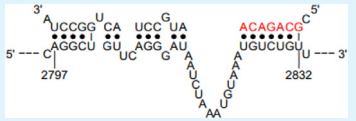
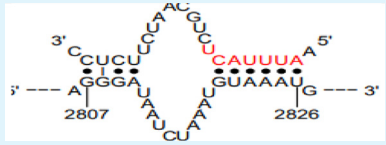
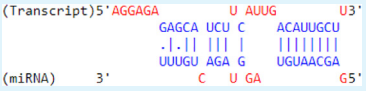
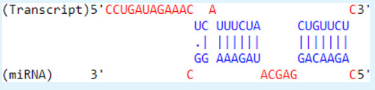
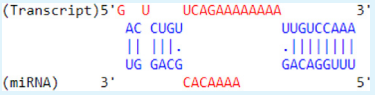
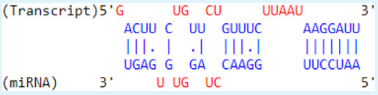
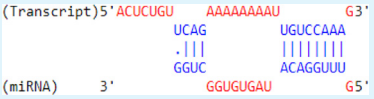
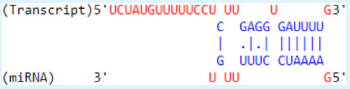
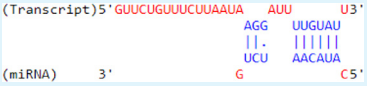
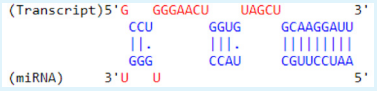
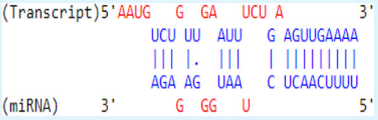
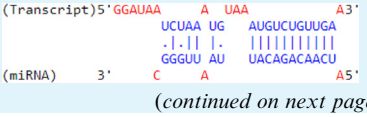
MicroRNAs	ACE2 Gene Sequences	SNPs	Databases	Screenshots
Hsa-miR-6806-5p	TGTAGGCAAATC[A*/G]CCATA*GTACTTGA*ACT	rs976149849A>G	DianaTool miRDB	
Hsa-miR-4766	ACTGAAAGCAATG[T/C]CAATGAA*GA*TGC*TCT	rs1436185384T>C	DianaTool miRDB	
Hsa-miR-4658	TT[G/C]AGTGTCTGCTTTGTGCTCAGCACTGC	rs1481257600G>C	DianaTool STarMirDB	
Hsa-miR-3684	TTAGACCTGTCA[C/A]CTTGAAGAAAGCATGA	rs986013272C>A	DianaTool STarMirDB	
Hsa-miR-3684	TTAGAC[C/T]TGTCACCTTGAAGAAAGCATGA	rs1178938686C>T	DianaTool STarMirDB	
hsa−miR−3653	TTAAGAAA*[C/T]AGAACAGAAGT	rs1423809569C>T	STarMirDB	
hsa−miR−3166	AA*CAGACATTTACATTTAGATTAT[C/T]CCTGAA*CAGCCTG	rs1325998023C>T	STarMirDB	
hsa−miR−4699−3p	CATTTACATTTAGATTAT[C/T]C*CT	rs1325998023C>T	STarMirDB	

Table 2: List of miRNAs possessing SNPs in their genomic sequences at target sites in the 3′ UTR of *ACE2*.

microRNAs	MicroRNAs Sequence	SNPs	Screenshot
hsa-mir-3908	GAGCAATGTAGG[T/-]AGACTGT*TT*	rs1450533585 T>-	
hsa-mir-4773-2	CAGA[A/G]CAGGAGCATAGAAAGG*C	rs370179731 A>G	
hsa-mir-3065	TCAACAAAATCACTGATG*CTG*[G/C]A	rs1251048260 G>C	
hsa-mir-4520b	TTTGGA[C/G]AG*AAAACACG*CAGGT	rs1238432232 C>G	
hsa-mir-632	GT[G/C]TCTGCTTCCTGTGG	rs1317052508 G>C	
hsa-mir-362	AATCCTTGG*AACCTAGG*TGTG*[A/T]GT	rs374141752 A>T	
hsa-mir-7850	GTTTGGACAT[A/T]GTGTGGCTGG*	rs902278877 A>T	
hsa-mir-4668	GAAAATCC[-/TTTTTT]T*TT*TTGT	rs1394117376 ->TTTTTT	
hsa-mir-6755	TGTTGTCATG[-/T]TTTTTTCCCTAG	rs1314065238 ->T	
hsa-mir-4666	CATA[C/A]AAT*CTG	rs958857049 C>A	
hsa-mir-500b	AATCCT[T/A]GCT*ACCTG*GGT	rs781999368 T>A	
hsa-mir-1305	TTTTCAACTCT[A/G]ATGGG*AGAGA	rs985999312 A>G	
hsa-mir-421	GC*CC*AATTA*ATGTCTG[T/C]TGAT	rs1345251789 T>C	

(*continued on next page*)

Table 2 (*continued*)

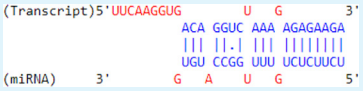
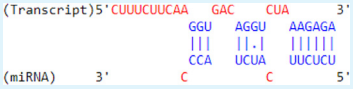
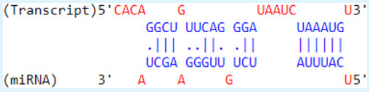
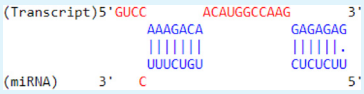
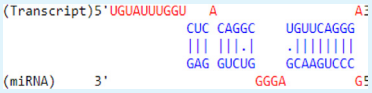
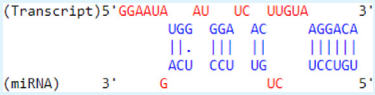
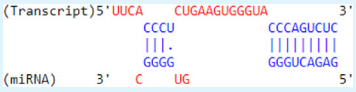
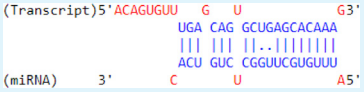
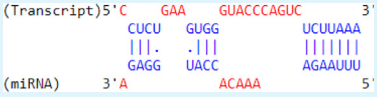
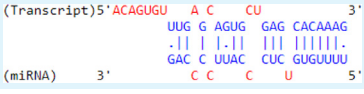
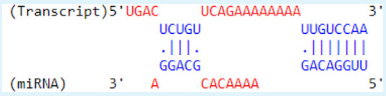
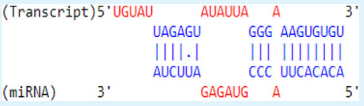
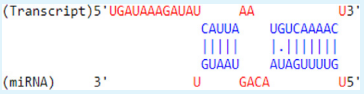
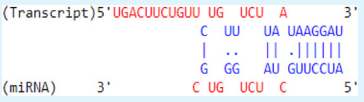
microRNAs	MicroRNAs Sequence	SNPs	Screenshot
hsa-mir-3909	TGTCCTCTAG[G/A]GCCTGCAGTCT	rs1234585731 G>A	
hsa-mir-942	TCTTCTCTGTTT[T/C]GG*CCATGTG	rs1384667146 T>C	
hsa-mir-6515	TCTCTTC[A/C/G]T*CTACCC	rs769602728 A>C,G	
hsa-mir-574	TGAGTGTGTGTGTGTG[A/T]GTGTGT*	rs1309447183 A>T	
hsa-mir-4729	TCATTTATCT*GT*TG[G*/C]G*AAGCT*A	rs1167550125 G>C	
hsa-mir-6873	GAAAGACAGA[G/A/C]AGAA*	rs554964784 G>A,C	
hsa-mir-345	GCCCTGAACG*A[G/T]GGGT*CTGGAG	rs1227054156 G>T	
hsa-mir-6852	CTGA*GGAACAG[A/C]GGACA	rs1221759458 A>C	
hsa-mir-4463	GAGACTGG[G/A]GTG*GGGC	rs991713068 G>A	
hsa-mir-2113	ATTTGTGCTT[G*/C]G*CTCTGTCAC	rs1205511802G>C	
hsa-mir-200c	TAATACTGCCG*G*GT*AA[T/C/G]GATGG*A	rs1400433260 T>C,G	
hsa-mir-3658	TTTAAGAAAACAC[C/T]T*G*GAGA	rs1024225815 C>T	
hsa-mir-5010	T*TTTGTGTCT[C/T]CCAT*TCCCCAG*	rs374388783 C>T	

Table 2 (*continued*)

microRNAs	MicroRNAs Sequence	SNPs	Screenshot
hsa-mir-4520b	TTGGA[C/G]AG*AAAACACG*CAGG*	rs1238432232 C>G	
hsa-mir-216b	TAGAA*TCTCTA[C/T]GGGTAAGTGTGT	rs368125248 C>T	
hsa-mir-2052	TGTTTTGAT*A[A/G]CAGTAATGT	rs1468783220 A>G	
hsa-mir-502	ATCCTTG*CTAT[C/G]TG*G*GTGC	rs1557183210 C>G	

Results

In the in-silico part of this study, we used different tools (Supplementary Data) and identified 80 SNPs in the 3′ UTR of *ACE2* that were associated with 30 SNPs of different miRNAs and might target their mirSNPs at their binding sites to increase or decrease affinity to create damage (Tables 1 and 2). Interestingly, we found that the binding site of miR-3658, which interacts with then 3′ UTR of *ACE2*, featured three SNPs (rs1457913029, C>T; rs960535757, A>C, G; rs1423809569, C>T), and that the genomic region sequence has a single SNP (rs1024225815, C>T) with the same nucleotide variation to rs1457913029, C>T (Figure 1) that might elevate infection susceptibility.

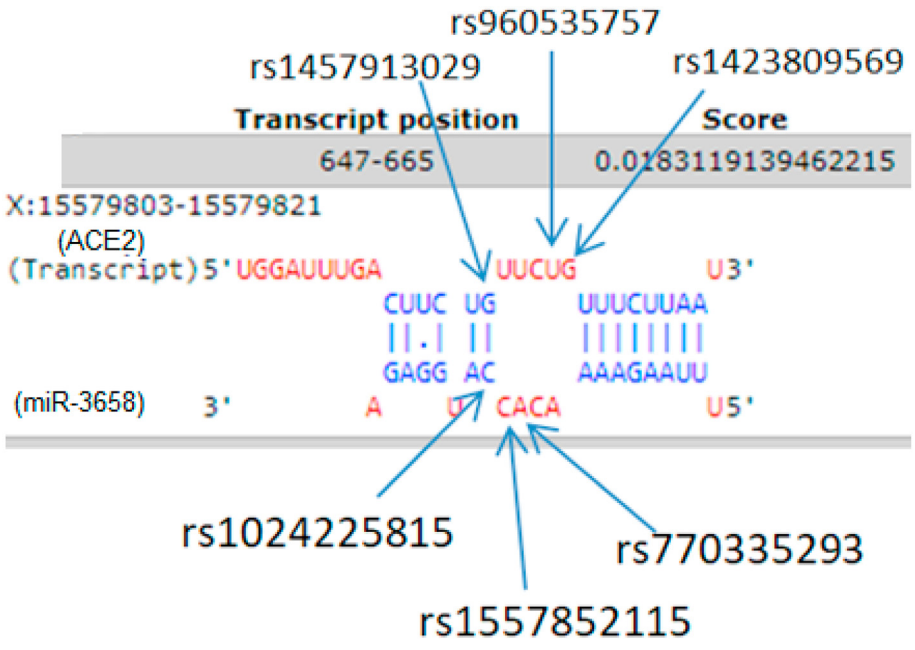


Figure 1: Six important SNPs were detected at the binding sites between miR-3658p and the *ACE2 gene*. Interestingly, the binding site for miR-3658 on *ACE2* features three SNPs (rs1457913029, C>T; rs960535757, A>C, G; rs1423809569, C>T) on the *ACE2* 3′ UTR. Furthermore, the genomic sequence possessed an SNP (rs1024225815, C>T) at the same nucleotide with rs1457913029. Similarly, three other SNPs (rs1557852115, C>G; rs770335293, A>G; rs1024225815, C>T) were identified at the first binding site of miR-3658. miRSNP (rs1457913029), located at the *ACE2* 3′ UTR, and the rs1024225815 SNP at miR-3658 in the genomic sequence cross-matched at the same binding site.

Table 3: List of hypertension-related genes (other than *ACE2*) targeting miR-3658 and associated with hypertension pathways that are susceptible to SARS-CoV-2 infection.

Sr#	Genes	Relationship with hypertension	References
1.	*TMPRSS Family*	Interactions with ACE2 and serine protease TMPRSS2 to facilitate the entry of SARS-COV-2	11
2.	*ADAM17, ADAM10 &* ITGB1	ACE2 can be regulated by shedders (ADAM10, ADAM17) by interacting with integrins (ITGB1)	26
3	*TLR4*	Highly variable in Irani Populations during SARS-CoV-2 infection	31,32
4	*SLC6A19*	Functional association of mutant *SLC6A19* transporters with ACE2 in the intestine	28
5	*IL-6 & chemokine (C−C motif) ligand 5*	*ACE2* KO hypertensive mice showed enhancement of pro-inflammatory cytokines, i.e., IL6, and chemokine (C −C motif) ligand 5	29,30

Similarly, we also detected three other SNPs (rs1557852115, C>G; rs770335293, A>G; rs1024225815, C>T) at miRNA-3658 binding sites within the first position. The genomic sequence was cross matched for similar sites in the binding region for miRSNP (rs1457913029) in the 3′ UTR of *ACE2* and SNP (rs1024225815) in miR-3658 (Figure 1) that might exert a significant impact on the severity of COVID-19 in hypertensive individuals.

In the second part of the study, qRT-PCR analysis identified significantly lower expression levels of *ACE2* in healthy controls as compared to patients ($p < 0.05$). We hypothesize that miR-3658 targeting of the *ACE2* gene can inhibit complications pertaining to hypertension with SARS-CoV-2 infection (Table 4). Computational analysis revealed

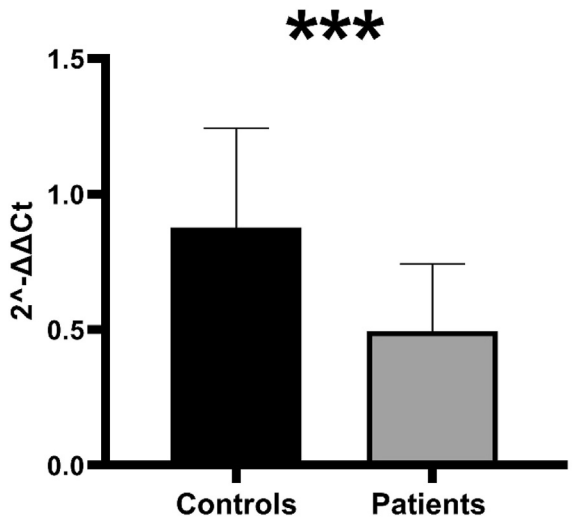


Figure 2: Differences in *ACE2* expression between in healthy controls and patients (nasopharyngeal swab samples) as normalized by the *GAPDH* gene ($p < 0.05$). Data are expressed as mean ± SD. Data were non-parametric and were analyzed by the Mann−Whitney U test.

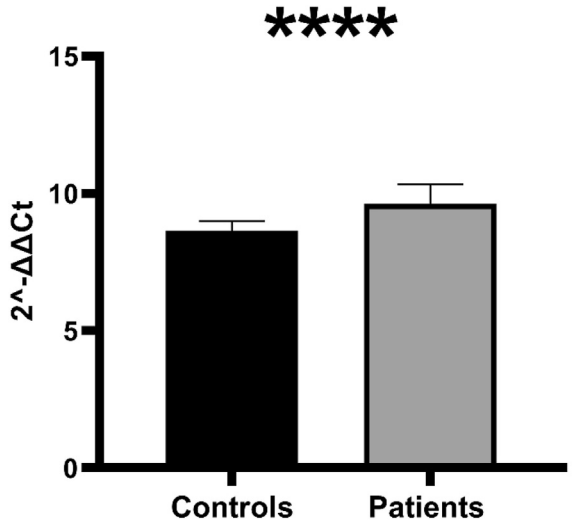


Figure 3: Differences in miR-3658 expression between healthy controls and COVID-19 patients (nasopharyngeal swab samples) as normalized by the RNU6B_13 gene ($p < 0.05$). Data are given as mean ± SD. Data were non-parametric and analyzed by the Mann−Whitney U test.

Table 4: Differences in *ACE2* and miR-3658 gene expression between healthy controls and patients (nasopharyngeal swab samples) as normalized by *GAPDH* and RNU6B genes.

Variables	Controls Mean ± SD	Patients Mean ± SD	*P*-value
2^-ΔΔCt (ACE2- GAPDH)Ct	0.877 ± 0.366	0.495 ± 0.248	0.0002
2^-ΔΔCt (miR-3658 - RNU6B_13)Ct	8.625 ± 0.362	9.627 ± 0.709	<0.0001

that miR-3658 targets the ACE2 3′ UTR region and that the *ACE2* gene partially inhibits transcription via miR-3658. It has yet to be demonstrated whether miR-3658 is associated with hypertension or SARS-COV-2 infection. The results of our in vitro study were further corroborated by the findings of the in-silico investigation (Figures 2 and 3).

Discussion

It is generally known that detecting binding and matching regions on miRNA is laborious and expensive. However, it is highly advantageous to use bioinformatics techniques to assess miRNA/target interactions prior to performing laboratory trials. Furthermore, researchers face difficulties when attempting to identify mysterious pathophysiological mechanisms used by infectious agents such as SARS-CoV-2. Thus, identifying miRNA-mediated target binding mechanisms might be useful when evaluating the severity of infections through computational analysis.[5,23,24] It is a key advantage that these targeted databases typically possess miRNAs-target interactions at 3′ UTR sites which represented our primary focus in this investigation.

The 3′ UTR regions of miRNAs and the *ACE2* gene were downloaded from selected databases (Supplementary Data) and compiled into a list to check mismatches and variations. Furthermore, the target sites were precisely predicted using direct or wobble matching with their related nucleotides.[25] We adopted the wobble theory, which explains why G matches to U are permitted in place of C.[25] Furthermore, SNPs were identified at the 3' UTR of ACE2 and a complete 80 MirSNPs list was created. The SNPs of targeted genes (*ACE2* and microRNAs) were scanned from NCBI at 3′ UTR regions and cross-matched SNPs in these targeted genes were manually determined.[5] Specific SNPs (matched and unmatched SNPs) were analyzed in depth for *ACE2*, which is known to be associated with hypertension and COVID-19 (Figure 1). The results of the in vitro study were corroborated by the findings of the in-silico investigation.[6]

It was previously reported that *ACE2* and SARS-COV-2 infections were directly related to hypertension lethality.[26] Furthermore, the results of our study are also in line with those of Pan et al. who reported the rs4646188 variant in *ACE2* as a potential diagnostic marker for hypertension, dyslipidemia, and ischemic stroke.[27] Moreover, the severity of SARS-COV-2 infection may be explicitly associated with ACE2 and *TMPRSS2* gene polymorphism.[11]

Similarly, other studies have also stated that interactions between *ACE*/*ACE2* and miRNAs in hypertensive patients might increase the possibilities of SARS-CoV-2 infection.[5,11,26,28–30] However, microRNAs, such as miR-145, miR-27a/27b, and miR-483-3p, can suppress *ACE*/*ACE2* expression patterns by increasing or minimizing their binding affinity at their corresponding binding sites in the 3′ UTR of *ACE*/*ACE2*.[11,26,28,29] In addition, we reported the association of miR-3658 with different *ACE2* genes for the first time. In this study, we found that miR-3658 can target numerous *ACE2*-related genes including *TMPRSS* family, *ADAM10, ADAM17*, ITGB1, *TLR4, SLC6A19, IL-6, and chemokine Chemokine (C-C motif) ligand 5 (C—C motif) ligand 5*) (Table 3) that have already been linked to hypertension and COVID-19.[5,11,26,28–33]

Multiple databases (Supplementary Data) have reported allele frequencies of distinct SNPs in the 3′ UTR region of *ACE2* and microRNAs in the National Center for Biotechnology Information (NCBI). However, in this study, we documented the allele frequencies of informative SNPs in the 3′ UTR region of *ACE2* and microRNAs in different populations (Supplementary Data) that might be used as potential markers and therapeutic targets.[27,34] Regardless of these documented SNPs in the 3′ UTR region of the *ACE2* gene or miRNAs, it is still unknown which SNPs are functional and informative. Our study provides inclusive insight regarding miRSNPs (rs1457913029) at the *ACE2* 3′ UTR and the miR-3658 genomic sequence (rs1024225815) which were cross-matched at the same site of the binding region (Figure 1). Furthermore, our in vitro study identified striking genetic variations. There are several limitations to the current understanding of the relationship between the 3′ UTR of the *ACE2* gene and miR-3658 that elevate the susceptibility and severity of COVID-19. Some of these limitations include:

1. The relationship between the 3′ UTR and COVID-19 susceptibility and severity is complex and influenced by multiple factors. Genetic variations in the 3′ UTR of the *ACE2* gene are just one of the many factors that can affect susceptibility and severity of COVID-19.
2. This study was small sample size which can limit the generalization of the results to the broader population.
3. The majority of the study was done on individuals from a specific race or ethnicity and the findings might not be applicable to other populations.
4. This study did not take into account the effect of comorbidities, which can affect the susceptibility and severity of COVID-19.
5. The study was reported conflicting results, which makes it difficult to draw definitive conclusions about the relationship between the 3′ UTR and COVID-19 susceptibility and severity.
6. The study was focused on a single variation in the 3′ UTR of the *ACE2* gene and do not take into account other genetic variations in the *ACE2* gene or other genes that may also play a role in susceptibility and severity of COVID-19.

It is important to note that these limitations do not negate the importance of the findings, but rather highlight the need for more research to fully understand the relationship between the 3′ UTR of the *ACE2* gene and miR-3658 and susceptibility and severity of COVID-19.

Conclusion

Our study provides inclusive insight regarding the SNPs of miRSNP (rs1457913029) at *ACE2* 3′ UTR and the miR-3658 genomic sequence (rs1024225815) which were cross-matched in the binding region for same sites and striking findings regarding genetic variations via in vitro study. Informative SNPs are of great value owing to the functional effect of miRSNPs/variations in hypertension and correspondingly their potential association with COVID-19 patients (in vitro) and computational analysis (in silico). This study provided novel findings related to the effects of

miRSNPs on the *ACE2* gene and SNPs in miRNA genes targeting 3′ UTR that are associated with hypertension and SARS-COV-2 infection validated via in silico and in vitro analysis. The findings of this study might help in the development of novel medications for COVID-19 patients, specifically addressing the function of the 3′ UTR region of ACE2 and the correlation of COVID-19 with severity.

Availability of supporting data

All data presented in the manuscript and the supplementary data are available upon request.

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Conflict of interest

The authors have no conflicts of interest to declare.

Ethical approval

The study was performed in accordance with the Ethical Committee of research involving humans. Ethical oversight and approval were obtained by the Review Ethical Committee (Regr/Admin/10) with Oric No. 169, dated 07-01-2020. From August 2020 to August 2021, a total of 200 nasopharyngeal/mouth swabs samples were collected from Multan, Pakistan.

Consent

Written consent was taken from all patients and approved by the university.

Authors' contribution

The authors have declared the following contributions: concepts and design by MS, MSK and SSK; experiments performed by MSK, YJ, and MS, FS, UY, MN; data analyzed by MS; tables and figures prepared by MS; manuscript written by SSK and MS. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtumed.2023.02.009.

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