Indonesian Journal of Pharmacy

VOL 34 (4) 2023: 640–650 | RESEARCH ARTICLE

Detection of ACE Gene SNPs Using rhAmp Genotyping Platform and Their Association with I/D Polymorphism in COVID-19 Patients with Hypertension

Ingrid Faustine[1,6], Amarila Malik[2*], Retnosari Andrajati[3,4], and Septelia Inawati Wanandi[5]

1 Faculty of Pharmacy, Universitas Indonesia, Depok, 16424, West Java, Indonesia
2 Division of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmacy, Universitas Indonesia, Depok, 16424, West Java, Indonesia
3 Division of Clinical Pharmacy, Faculty of Pharmacy, Universitas Indonesia, Depok, 16424, West Java, Indonesia
4 Universitas Indonesia Hospital, Jl. Prof. Dr. Bahder Djohan, Pondok Cina, Depok 16424, West Java, Indonesia
5 Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia, Central Jakarta, 10430, DKI Jakarta, Indonesia
6 Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Tadulako University, Palu, 94148, Central Sulawesi, Indonesia

Article Info

Submitted: 17-09-2022
Revised: 20-02-2023
Accepted: 24-05-2023

*Corresponding author
Amarila Malik

Email:
amarila.malik@ui.ac.id

ABSTRACT

The high activity of Angiotensin Converting Enzyme (ACE) in patients with hypertension (HT) potentially affects the risk and severity of COVID-19 infection. The ACE gene variation leads to the modification of ACE activity in the Renin-Angiotensin System (RAS). Therefore, this study aims to identify three ACE gene SNPs using the rhAmp SNP genotyping platform, i.e., rs4331 (A/G), rs4341 (G/C), and rs4343 (G/A), as well the linkage disequilibrium (LD) with rs1799752 (I/D) polymorphism. It also analyzed their associations with COVID-19 severity and comorbid HT. This cross-sectional study was carried out in two regions of Indonesia, i.e., Palu City, Sulawesi, and Lahat District, Sumatera, each representing the eastern and western parts, with a total of 95 COVID-19 patients recruited in 2021. The SNP detection results showed that the percentage of genotypes GG, AG, and AA, i.e., 57%, 32%, and 11%, respectively, at rs4331, corresponded with II, DI, and DD at rs1799752. However, at rs4341, only genotype GG was found, while at rs4343, 37% of the samples were undetermined. The results also revealed that rs4331, rs1799752, and rs4343 showed strong LD (D'= 0.97 and 0.99), while rs4331 and rs1799752 were considered replaceable and can serve as gene markers (R^2= 0.957). Genotype rs4343 and haplotype analysis showed a significant difference between the residents of western and eastern Indonesia ($p< 0.05$), while no difference was observed for comorbid HT and the severity of COVID-19. About 17% of the G-I-G-G haplotypes indicated moderate-severe (M-S) severity, while 15% of the A-D-G-G haplotypes showed mild (M) severity. It was concluded that the rs4331, rs4343, and rs4341 genotypes could successfully be detected by using the rhAmp genotyping platform. However, the association with I/D polymorphism was likely co-inherited for rs4331 and rs4343 only. The two dominant haplotypes associated with mild and moderate-severe symptoms were A-D-G-G and G-I-G-G, respectively.

Keywords: ACE, COVID-19, Hypertension, Indonesia, rhAmp SNP genotyping

INTRODUCTION

Health workers have continued to face various challenges in the management of COVID-19. Although vaccination has been implemented, SARS-CoV-2 genetic mutation continuously occurs, increasing the risk for infection. There have been 547 million cases of COVID-19 globally, and South-East Asia is the region with the fourth highest

number of cases after Europe, America, and the Western Pacific ("WHO Coronavirus (COVID-19), 2022). Indonesia is one of the countries in South-East Asia with 6 million confirmed cases, 49.7% of which have comorbid hypertension (HT) ("Peta Sebaran | Covid19, 2022). In addition, the country has a mortality rate of 2.6%, which is higher than the global percentage of only 1.1% ("WHO Coronavirus (COVID-19), 2022).

Blood pressure is regulated by the renin-angiotensin system (RAS) through the conversion of angiotensin I to angiotensin II, with the Angiotensin Converting Enzyme (ACE) playing a crucial role (Wong, 2016). ACE2, a homolog of ACE in the RAS, is recognized as the gateway for SARS-CoV-2 infection in humans (Sabater Molina et al., 2022). In patients with HT, Angiotensin I (Ang-I) levels are often elevated, leading to changes in ACE and ACE2 activity (Devaux et al., 2020). This affects individual vulnerability to COVID-19 exposure and can contribute to developing more severe symptoms (Calabrese et al., 2021). At the genomic level, the insertion/deletion (I/D) polymorphism of the ACE gene in intron 16, containing 287 base pairs (bp) of Alu element, affects plasma levels and ACE activity, thereby modulating susceptibility to HT (Kang et al., 2012; Bánhegyi et al., 2021).

Among the Caucasian population, genotype DD is reportedly predominant and is more susceptible to cardiovascular disease as well as COVID-19 than genotype DI or II (Calabrese et al., 2021; Han et al., 2017). In Asian populations, genotype II is dominant, and there is no association between I/D polymorphism and cardiovascular diseases (Alexander et al., 2020; Krishnan et al., 2016). Despite some conflicting findings, several studies have concluded that the I/D polymorphism of the ACE gene serves as a genetic marker for the development of cardiovascular disease (Heidari et al., 2019). Furthermore, the presence of I/D polymorphisms in introns suggests that other functional polymorphisms could also influence the control of ACE transcription and its enzymatic activity (Kankanit et al., 2018; Peplonska et al., 2017). The detection of polymorphism has been supported by various methods. For example, the conventional genotyping method was used for screening ACE polymorphisms which consists of two steps, polymerase chain reaction (PCR) and agarose gel electrophoresis, but these steps are quite time-consuming (Heidari, Hadadzadeh, and Fallahzadeh, 2019). Therefore, fast and reliable alternative methods are needed to detect other forms of mutations, such as rhAmp SNP genotyping for large sample studies (Beltz et al., 2018).

The fine mapping analysis of the ACE gene involving I/D polymorphisms and 31 SNPs showed that the region between exon 13 and intron 18 was most associated with ACE activity (Chung et al., 2013). Given its importance in the pathogenesis of HT, the ACE gene is considered a good candidate for COVID-19 severity studies, but population differences can also lead to different phenotypes of I/D genotypes. Therefore, this study aims to identify the linkage disequilibrium of SNPs at three rs number with I/D polymorphisms as well as their associations with the severity of COVID-19 and comorbid HT in the Indonesian population.

MATERIALS AND METHODS

Study design, participants, and blood samples

This was an observational cross-sectional study involving 95 subjects diagnosed with COVID-19 in 2021 in the Palu City-Central Sulawesi and Lahat District-South Sumatra, representing the population of eastern and western Indonesia. The inclusion criteria were natives or residents who had settled in the area and were more than 18 years old. COVID-19 results were obtained from patients who were positive for SARS-CoV-2 with real-time-Polymerase Chain Reaction (rt-PCR) test. Regarding Hypertension (HT), the patients recruited were those with a history of HT in the medical record and receiving therapy during COVID-19 treatment at Central Sulawesi and Lahat district general hospitals. Patients undergoing self-quarantine under the supervision of the Lahat District Public Health Center were also included. Blood samples were collected by trained laboratory personnel from all subjects with informed consent. Subjects discharged at their request or did not have complete medical records were excluded.

DNA Extraction

Blood samples obtained in EDTA tubes were stored at 4°C in the laboratory. Genomic DNA was extracted from 2 ml whole blood using the kit and protocol on the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). Total DNA was measured spectrophotometrically using a NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, USA) at an absorbance of 260/280nm. The extracted DNA was stored at -20°C for further use in the next stage.

Determination of ACE I/D Genotype

The conventional PCR was performed to detect the presence or absence of Alu element insertion in intron 16 of the ACE gene at 1799752 (Krishnan et al., 2016). Furthermore, the forward and reverse primer pair, i.e., 5'-CTGGAGACCACTCCCATCCTTTCT-3' and 5'-GATGTGGCCATCACATTCGTCAGAT-3', was synthesized by IDT (Integrated DNA Technologies, USA). The PCR fragment consisted of three genotypes, including a 490 bp band (II), a 190 bp band (DD), as well as both a 190 and 490 bp band (DI).

Genotyping of SNPs by rhAmp

SNPs were selected from the NCBI (March 2021) database, and selection criteria included: being tested and validated experimentally and spanning the region around ACE I/D (58,919,622 bp). With these criteria, three SNPs from the ACE gene were selected to design the rhAmp SNP Genotyping assay, namely rs4331 (A/G) exon 15, rs4341 (G/C) intron 16, and rs4343 (G/A) exon 17 (Glenn et al., 2008; *ACE gene NCBI*, 2021; *rs4343 - SNPedia*, 2021; *rs4341 - SNPedia*, 2021)

DNA samples were diluted with TE buffer pH 7.5 to a concentration of 3 ng/µl. Afterward, SNP genotyping was performed using rhAmp SNP Assays, rhAmp Genotyping Master Mix, and rhAmp Reporter Mix with or without passive reference dyes (www.idtdna.com/rhAmp-Genotyping).

The reaction contained a 5.3 µL of master mix genotype rhAmp SNP, 0.5 µLrhAmp Assay, and 2 ng of gDNA. Moreover, gBlock was used as a positive control, and nuclease-free water as NTC (non-template control). The reactions of the rhAmp SNP genotypes were run on an AriaMx instrument (Agilent, Santa Clara, USA) with thermal cycling of 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 60°C for 30 s, and 68°C for 20 seconds per published protocol (Integrated DNA Technologies Inc, 2017). Call rate was defined as the percentage of sample calls with an assigned SNP genotype, while accuracy was the percentage in a given genotype. Reported call rates and accuracy were determined using automated calls provided by the AriaMX Real-Time PCR Software for each of the 95 samples with a total of 285 tests.

PCR and Sanger sequencing

Samples that were genotyped ambiguously by the rhAmp SNP genotyping assay were subjected to sequencing analysis. The PCR primers were designed using the PrimerQuest® and Oligoanalyzer tools program by Integrated DNA Technologies (https://sg.idtdna.com). The primer pairs generated approximately 700 base pairs containing three SNP at rs1799752, rs4341, and rs4343. The ACE primer used was 5'-GGAGAGGAGAGAGACTCAAG-3' as the forward and 5'-GACCCAAGTGCCAGTGATG-3' as the reverse. A total of 6 samples were sequenced with genotype II traits at rs1799752 and ambiguous genotypes at rs4343.

PCR reaction of 50 µL volume was carried with 8.2 µl ddH2O, 25 µL KOD Fx Neo buffer (Toyobo, Osaka, Japan), 4 µL (20 ng/µL) DNA template, 10 µL dNTP (Toyobo, Osaka, Japan), 1 µL of each primer, and 0.8 µL KOD Polymerase (Toyobo, Osaka, Japan). Amplification was performed with T-Professional Basic Thermocycler (Biometra, Ltd., Jena, Germany). The protocol included predenaturation at 95°C for 1.5 minutes, followed by 35 cycles at 94°C for 30 seconds, annealing at 58°C for 15 s, and extension at 68°C for 45 s. The PCR amplicon was further processed through a Sanger sequencing conducted by 1st BASE, Apical Scientific (Selangor, Malaysia). The obtained sequences were checked for the presence of polymorphisms using the software Sequence Scanner 2 (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA) and Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Ethical approval

All methods were carried out according to the relevant guidelines and regulations. Furthermore, the experimental protocols followed were approved by the ethics committee of the Faculty of Medicine, the University of Tadulako, as well as the University of Indonesia Hospital (RSUI) with the approval number 7916/UN.28.1.30/KL/2020 and 0058/SKPE/KKO/2021/0, respectively. Informed consent was also obtained from all subjects.

Data analyses

Categorical variables were expressed as amounts and percentages, while differences between groups were evaluated by Chi-square and Fisher's test using SPSS 21 statistical analysis (IBM Corp., Armonk, NY, USA). The standard statistical significance was $p < 0.05$, and all p-values were two-sided. The association between SNP, I/D, and haplotype with response was used in haplotype analysis in the SNPStats application (https://www.snpstats.net/analyzer.php).

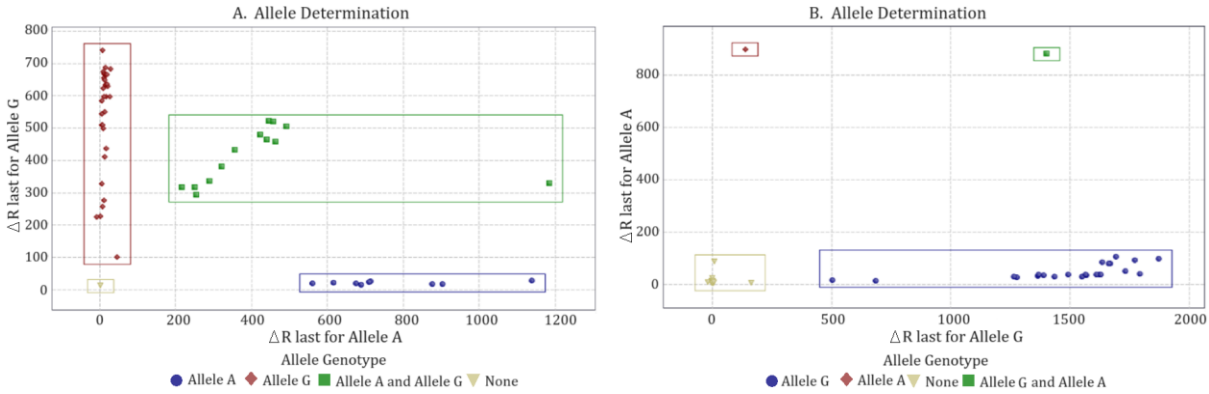

Figure 1. Examples of allelic discrimination plots of rhAmp SNP Genotyping. The vertical and horizontal axes represent the fluorescence intensity for FAM and VIC, respectively. 1A: beige denotes the reaction product without DNA (negative control), blue denotes the wildtype homozygous genotype, green denotes the heterozygous genotype, and red denotes the mutant homozygous genotype. Yellow= NTC. 1B: The plot marked by the black circle was unamplified due to the 287 bp insertion at rs1799752 and was considered an ambiguous genotype.

RESULTS AND DISCUSSION

Detection of ACE Gene SNPs

Thirty-one SNPs were reported in LD with I/D polymorphism (Chung et al., 2013). Three of these were investigated in this study. One of those SNPs, rs4343, occurred in the upstream region in exon 15. In comparison, the other two, rs4341 and rs4343, were located in the downstream region in intron 16 (Figure S1). These SNPs were detected using the rhAmp SNP genotyping method, along with the RT-PCR tool, following the protocol guidelines from IDT (Integrated DNA Technologies, USA).

The output of rhAmp SNP results was presented in allele determination with four colors representing three genotypes, namely homozygous wildtype, heterozygous, homozygous mutant, as well as a negative control (NTC) (Figure 1). Figure 1A shows that the allelic discrimination plot for all samples exhibited a high signal with good and homogeneous genotype cluster separation for rhAmp SNP testing. Overall, the results of the rhAmp SNP test performance showed a 92% call rate and 59% accuracy, according to the genotype published in the NCBI database. Low accuracy was found in rs4341 and rs4343, which can be attributed to their location downstream insertion site in intron 16 of the ACE gene. This result is illustrated in the allelic discrimination plot (Figure 1B), where a group of samples exhibited an ambiguous genotype indicated by plot position overlapping with the negative control plot (NTC).

Genotype Frequency of ACE Gene SNPs and I/D

This study collected samples from two locations, namely Lahat District – South Sumatra and Palu City – Central Sulawesi, representing western and eastern Indonesia, respectively. The results showed a significant difference between both populations at rs4343 ($p = 0.028$) (Figure 2).

The Palu population had the dominant genotype GG, while the genotype AA (12%) was predominant among the Lahat population. For rs4331 and rs1799752, wildtype homozygous genotypes, AA and DD, had the lowest percentage, while the homozygous mutants, GG and II, possessed the most significant portion. The detection of rs4341 in both populations revealed that all samples possessed the wildtype genotype, GG. Regarding rs4343, 37% of the samples were included in the ambiguous genotype (undetermined) because they did not match the three genotypes detected, namely GG, GA, and AA. Meanwhile, 57% had a homozygous wildtype genotype, GG, and 6% were classified as homozygous mutant AA.

Sanger sequencing was performed on genotype II with the ambiguous genotype at rs4343. The result revealed that all samples had an insertion of approximately 287 bp in length on intron 16, known as the Alu element sequence (Figure 3A). This insertion did not cause a mutation in rs4341 which remained as G (sense strand), but a mutation in rs4341 resulted in a change to A.

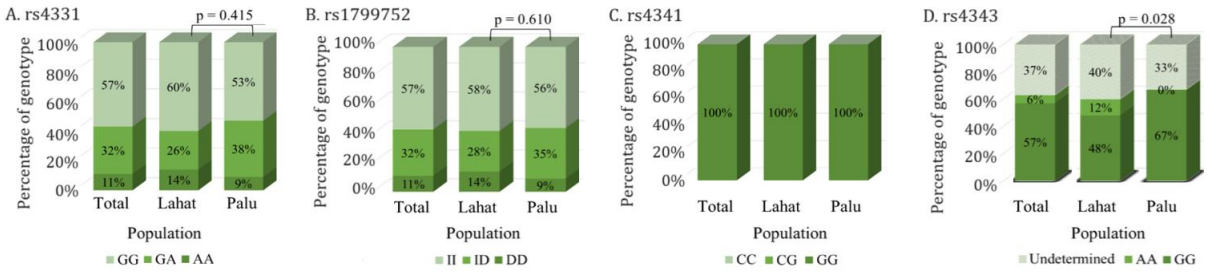

Figure 2. Overview of the I/D genotype and SNPs of the ACE gene in the Lahat and Palu populations. The darker green color represents the wildtype homozygous genotype, and the light green represents the mutant homozygous genotype of each rs number, A. rs4331, B. rs1799752, C. rs4341, and D. rs4343. The bar chart patterned at rs4343 (D) is an ambiguous genotype sample

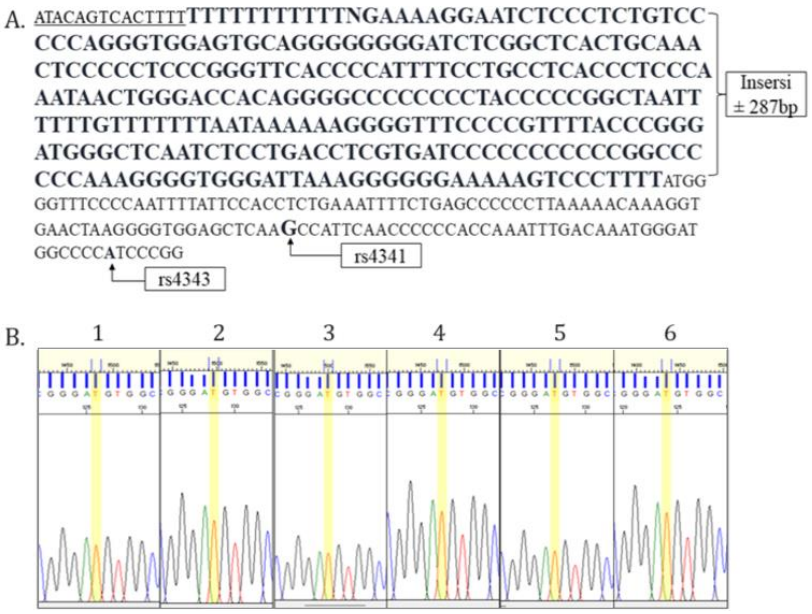

Figure 3. Two-way sequencing Sanger outputs on intron 16 and Exon 17 of the ACE gene. 3A: Shows the forward sequence where the base with the underline was the target insertion sequence, while the base sequence in bold was the insertion sequence of the Alu element at rs1799752 and the detected SNPs rs4341 and rs4343. 3B: Snapshot Sanger sequencing result ad rs4343 of 6 samples with ambiguous genotype, as an antisense strand.

Figure 3B shows G was greater than A in the antisense strand of rs4343 based on Sanger sequencing results.

Association of ACE Gene SNPs with I/D

Table I presents the seven models of genotypic variation obtained from polymorphisms in the ACE gene. Subjects with insertions in both alleles (genotype II) had homozygous mutations in the upstream region (rs4331), and 65% had ambiguous genotypes at rs4343. In addition, 22% of the Palu population was found to have a GG genotype, while the Lahat population had the AA genotype, at rs4343, with a significant difference (p = 0.015). Among subjects with only one allele insertion (ID), 97% also showed a heterozygous genotype (GA) at rs4331, consisting of 93% and 100% in the Lahat and Palu population, respectively. About 7% or one subject in the Lahat population had an ID genotype but a GG genotype at rs4331. Meanwhile, rs4341 and rs4343 both showed homozygous wildtype (GG) genotypes. All subjects with genotype (DD) also had wildtype genotypes at rs4331, rs4341, and rs4343.

Table I. Percentage of ACE Genotype variant

Variation	Genotype				Genotype percentage			p
	A>G[a]	D>I[b]	G>C[c]	G>A[d]	Total n (%)	Lahat Population n (%)	Palu Population n (%)	
Insertion – Insertion								
1	GG	II	GG	UD	35 (65)	20 (69)	15 (60)	0.015
2	GG	II	GG	GG	12 (22)	3 (10)	9 (36)	
3	GG	II	GG	AA	6 (11)	6 (21)	0 (0)	
4	GA	II	GG	GG	1 (2)	0 (0)	1 (4)	
Insertion – Deletion								
5	GA	ID	GG	GG	29 (97)	13 (93)	16 (100)	0.467
6	GG	ID	GG	GG	1 (3)	1 (7)	0 (0)	
Deletion – Deletion								
7	AA	DD	GG	GG	11 (100)	7 (100)	4 (100)	NA

UD (Undetermined), [a] rs4331, [b] rs1799752, [c] rs4341, [d] rs4343

Table II. Allele Frequency and Pairwise Linkage Disequilibrium Coefficients Between ACE Polymorphism

ID	Allele frequency (Allele 1/ allele 2*)	Linkage disequilibrium coefficients (D′ and R^2)	
		rs4331	rs1799752
rs4331	52/138	-	-
rs1799752	52/138	0.9734 0.957	-
rs4343	12/108	0.9973 0.462	0.9973 0.462

*Mutant allele

In the Linkage disequilibrium (LD) coefficients, the first and second lines represent D′ (absolute value) as well as R square (R^2), respectively, with the range between 0 to 1. The SNP rs4341 was not assessed in the pairwise LD analysis because all samples showed only one form of the same genotype. However, based on the value of D', all three SNPs were found to have high LD (Table II), indicating a high probability of these mutations being inherited together. The R2 value of rs4331 and rs1799752 suggested that the two SNPs can serve as gene markers to replace one another, but this was not the case for rs4343.

Haplotype Association of ACE Gene with Population, Comorbidities, and Severity of COVID-19

A total of sixty samples were further identified genotypically for haplotype analysis, while 35 were excluded due to the ambiguous result at rs4343. Five haplotype models were found based on the variation of the I/D and 3 SNPs. The main haplotypes were G-I-G-G, A-D-G-G, and G-I-G-A, while G-D-G-G and A-D-G-G were rare. Overall, there was a significant difference in the frequency of haplotypes between the two populations (p= 0.005), while no significant associations were found between comorbid hypertension (HT) and the severity of COVID-19 (SI Table).

A tree diagram was created to determine the frequency of susceptibility to COVID-19 severity based on haplotype variations, population, and the presence of comorbid HT. The chart revealed that the G-I-G-G haplotype in the Palu population (PP), without comorbid HT, had the highest susceptibility to COVID-19 with moderate to severe severity (18%). Meanwhile, the ADGG haplotype in the Lahat population (LP) had the highest frequency with mild symptoms (15%). This study found two rare haplotype variants: A-I-G-G in the non-hypertensive group of Palu and G-D-G-G in the Lahat population. These two rare haplotype variants showed moderate-severe symptoms of COVID-19.

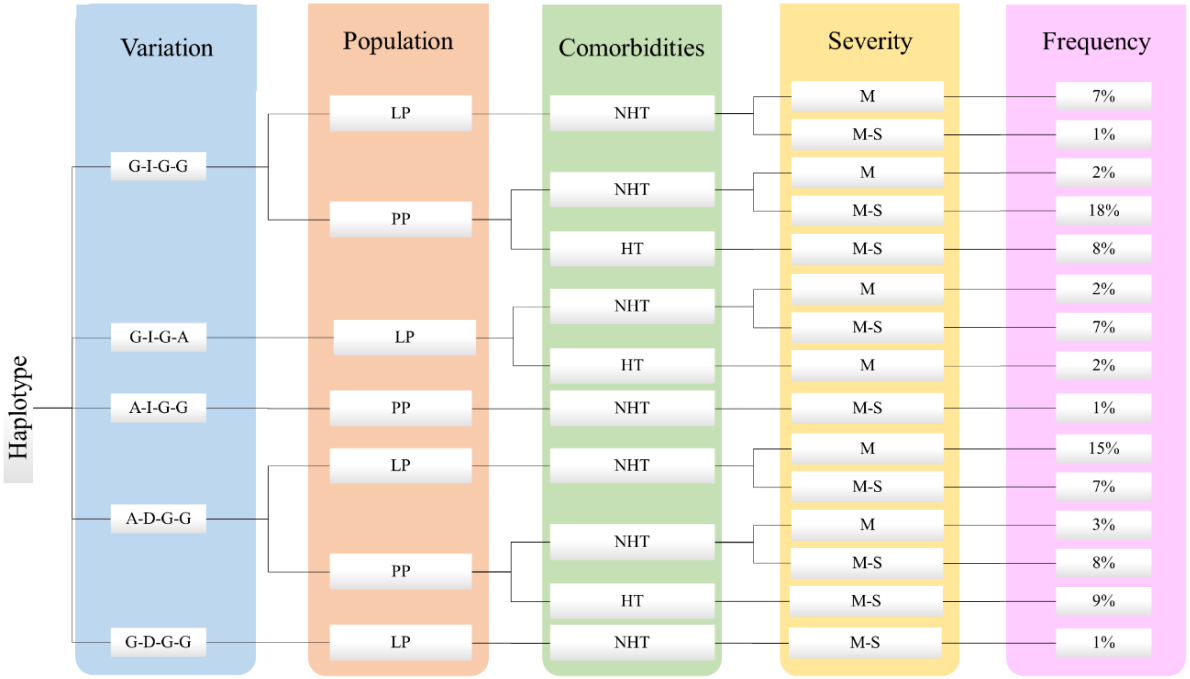

Figure 4. Frequency severity profile of Tree diagrams based on haplotype, population, and comorbid hypertension. Haplotype variation represents the decision note, population and comorbidities represent the event fork, and the proportion of severity represents the percentage of outcomes. LP (Lahat population), PP (Palu population), NHT (non-hypertension), HT (Hypertension), M (Mild), M-S (Moderate-severe)

Diversity of characteristics, lifestyles, and habits in the surrounding environment can lead to differences in gene expression among populations. The dominant population of Palu City and Lahat District consists of native people with a different socio-cultural life from the population on Java. For example, there are variations in lifestyle habits and food consumption. Previous studies showed that 43% of hospitalized COVID-19 patients in Palu City had comorbid hypertension (HT) (Faustine et al., 2021), while in Lahat, only 22.3% were found (Marteka et al., 2022).

ACE gene located on chromosome 17q23 and spans 21 kb, contains 26 exons. The presence or absence of the 287-bp Alu element sequence insertion in intron 16 determined the condition of genotypes II, DI, and DD. Polymorphisms in the Alu element and SNPs around the insertion region, such as the ACE I/D, are suitable markers for studying genetic variation in human populations (Chung et al., 2013). The distribution of the ACE genotype differed between races, and it was used as a marker in population structure analysis.

Alu element insertion was detected using conventional PCR amplification, and gel electrophoresis was marked in the 190 bp band for the D allele and 490 bp for the I allele, which served as a stable marker (Faustine, Unpublished). Another method used to detect polymorphisms was rhAmp SNP genotyping with rt-PCR. rhAmp SNP is a recently developed method compared to the previous two, namely Taqman and KASP. It showed better allele discrimination with almost the same percentage of call quality as the previous two methods (Ayalew et al., 2019). In addition, this method allowed for easy automated data handling, shorter processing times for larger samples, and a minimal DNA requirement of only six ng per test (Beltz et al., 2018). Among the three SNPs tested using rhAmp, rs4331 and rs4341 showed discriminatory allele results that could be well interpreted. For rs4343, approximately 37% of the samples had indeterminate genotypes, possibly due to the insertion of the 287 bp sequence in the upstream region.

(Glenn et al., 2009) assumed that the presence of an insertion in intron 16 would lead to a mutation in rs4341 (genotype CC) and rs4343 (genotype AA), but this study did not find consistent results. Sanger sequencing using confirm the SNP genotype was performed on intron 16 and exon 17 of the ACE gene in samples with

genotype II. Despite the insertion, the results showed that rs4341 still had base G (wildtype). In contrast, rs4343 exhibited base A (mutant), but the previous rhAmp method did not reveal an accurate genotype. The genotype discrepancy can be attributed to the accumulation of genomic alteration events in the ACE gene, leading to various forms of instability, which, in turn, caused differences in the phenotypic profile of each human (Melters et al., 2013).

Several studies have suggested that the variability in the genotypic distribution of the ACE polymorphism could explain the varying prevalence and clinical outcomes of COVID-19 among different populations worldwide. Two representative population studies were employed in Indonesia to analyze the role of ACE gene polymorphisms in comorbid HT in COVID-19 patients. The districts represent the western and eastern parts of the country, and results showed that 57% of the population experienced insertions in intron 16 at rs1799752 (genotype II). Aung (2020) and Bellone (2020) found that Asian and African populations with COVID-19 had genotype II, while the dominant Caucasian population had DI and DD genotypes (Aung et al., 2020; Bellone & Calvisi, 2020). Several reasons may account for the different results between these two populations. The first was the existence of epigenetic factors, such as DNA methylation and histone modification at the gene level. In addition, influence from lifestyle, environmental exposure, and socioeconomic status could have modulated gene function, causing differences in individual susceptibility to HT and COVID-19. Secondly, variations in genetic backgrounds in other races could have influenced the genotype (Han et al., 2017; Zeng et al., 2021).

Fluorescence separation by rt-PCR and LD pairwise calculations result between the SNPs showed that the three polymorphisms, rs4331, rs1799752, and rs4343, were inherited together. rs4331 was found to be the most suitable SNP for genotype I/D detection by the rhAmp method. This result was consistent with Bartakova's (2022) study stating that rs4331 and the I/D genotype, rs1799752 exhibited a strong relationship with one block based on the LD pairwise calculation (Bartakova et al., 2022).

Furthermore, subjects with insertions carrying the dominant G allele showed greater severity than deletion subjects, and they also had an A allele, as previously reported by (Alimoradi et al., 2022). I/D polymorphisms are known to alter circulating and tissue ACE concentrations. Genotype II was associated with lower serum ACE levels compared to genotype DI or DD (Kang et al., 2012; Bánhegyi et al., 2021). The low ACE activity was assumed to upsurge tissue ACE2 expression, which might explain the higher prevalence of COVID-19 infection and the tendency for increased severity (Yamamoto et al., 2020; Jacobs et al., 2021). In this study, the highest G-I-G-G haplotype with a frequency of 18% was found in the population of PL without comorbid HT, and they tended to experience moderate to severe COVID-19 severity. Meanwhile, the LP population without comorbid HT had the highest frequency of A-D-G-G and a mild COVID-19 severity with a frequency of 15%. This frequency was supported by a previous study regarding the association of comorbid HT with the severity and post-treatment conditions of COVID-19 patients in the eastern population. The patients had moderate to severe severity and a higher mortality rate than those without comorbid HT (Faustine et al., 2021).

Reliance on data obtained only from 2 representative Indonesian populations was the main limitation of this study. Some samples were also found to have comorbidities other than HT. However, these two populations could represent a geographically defined area with a high prevalence of individuals with HT, comorbidities, and mortality due to COVID-19. This is because the presence of other comorbid illnesses was offset by the fact that most of the severity associated with COVID-19 occurs in people with HT comorbid. Since Indonesia is a diverse country with multiethnic populations, the genotype data obtained from this study can have broader implications for several ethnic groups within the country. Although efforts were made to account for some potentially confounding variables, several factors, such as vaccination stage and HT therapy, could contribute to the differences in the COVID-19 severity profile. The results suggest that evaluating the genotypes rs4331, rs1799752, rs4341, and rs4343 could provide a new diagnostic approach for clinical assessment and risk management of COVID-19 patients with comorbid HT, allowing health workers to effectively and efficiently analyze the data.

CONCLUSION

Three ACE gene SNPs, namely rs4331, rs4343, and rs4341, were successfully detected from Palu City, Sulawesi, and Lahat District, Sumatera, using the rhAmp SNP genotyping method. The genotypes GG, AG, and AA percentages of 57%, 32%, and 11%, respectively, at

rs4331 corresponded with genotypes II, DI, and DD in polymorphism I/D. For rs4341, genotype GG was dominant, while at rs4343, approximately 37% of the samples were undetermined. Furthermore, only rs4331 and rs4343 were associated with I/D polymorphism as co-inherited markers, and the genotype of rs4343 was significantly different between Lahat and Palu populations. Two dominant haplotypes, A-D-G-G and G-I-G-G, were associated with mild (M) and moderate-severe (M-S) COVID-19 symptoms, respectively.

ACKNOWLEDGMENTS

We would like to thank the COVID-19 nursing and laboratory staff of the regional hospital at Lahat and Palu for contributing to this study.

CONFLICT OF INTEREST

The authors declare no competing interests.

FUNDING

This research was funded by the Ministry of Education, Culture, Research, and Technology, the Republic of Indonesia, via the grant scheme of Hibah Penelitian Disertasi Doktor, No.: NKB-884/UN2.RST/HKP.05.00/2022 to AM, partially funded by Universitas Indonesia via grant scheme Publikasi Terindeks Internasional Q2 (PUTI Q2) 2022-2023, No.: NKB-543/UN2.RST/HKP.05.00/2022 to AM. DM received a scholarship from Indonesia Endowment Fund for Education/LPDP (20200411021593) for a master study.

ADDITIONAL INFORMATION

Part of the results described in this study was presented at the 6th International Conference on Advance Pharmacy and Pharmaceutical Sciences (ICAPPS), October 27th-28th, 2022.

REFERENCES

ACE gene NCBI. (2021). Retrieved from https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=DetailsSearch&Term=1636

Alexander, V., Siregar, Y. and Arina, C. A. (2020). Correlation of ACE Gene Polymorphism and Hypertension in Stroke Ischemic Patients. *Sumatera Medical Journal*, 3(1), 41–47. https://doi.org/10.32734/sumej.v3i1.3310

Alimoradi, N., Sharqi, M., Firouzabadi, D., Sadeghi, M. M., Moezzi, M. I., & Firouzabadi, N. (2022). SNPs of ACE1 (rs4343) and ACE2 (rs2285666) genes are linked to SARS-CoV-2 infection but not with the severity of disease. *Virology journal*, *19*(1), 48. https://doi.org/10.1186/s12985-022-01782-6

Aung, A. K., Aitken, T., Teh, B. M., Yu, C., Ofori-Asenso, R., Chin, K. L., & Liew, D. (2020). Angiotensin converting enzyme genotypes and mortality from COVID-19: An ecological study. *The Journal of infection*, *81*(6), 961–965. https://doi.org/10.1016/j.jinf.2020.11.012

Ayalew, H., Tsang, P. W., Chu, C., Wang, J., Liu, S., Chen, C., & Ma, X. F. (2019). Comparison of TaqMan, KASP and rhAmp SNP genotyping platforms in hexaploid wheat. *PloS one*, *14*(5), e0217222. https://doi.org/10.1371/journal.pone.0217222

Bánhegyi, V., Enyedi, A., Fülöp, G. Á., Oláh, A., Siket, I. M., Váradi, C., Bottyán, K., Lódi, M., Csongrádi, A., Umar, A. J., Fagyas, M., Czuriga, D., Édes, I., Pólos, M., Merkely, B., Csanádi, Z., Papp, Z., Szabó, G., Radovits, T., Takács, I., … Tóth, A. (2021). Human Tissue Angiotensin Converting Enzyme (ACE) Activity Is Regulated by Genetic Polymorphisms, Posttranslational Modifications, Endogenous Inhibitors and Secretion in the Serum, Lungs and Heart. *Cells*, *10*(7), 1708. https://doi.org/10.3390/cells10071708

Bartakova, J., Deissova, T., Slezakova, S., Bartova, J., Petanova, J., Kuklinek, P., Fassmann, A., Borilova Linhartova, P., Dušek, L., & Izakovicova Holla, L. (2022). Association of the angiotensin I converting enzyme (ACE) gene polymorphisms with recurrent aphthous stomatitis in the Czech population: case-control study. *BMC oral health*, *22*(1), 80. https://doi.org/10.1186/s12903-022-02115-3

Bellone, M., & Calvisi, S. L. (2020). ACE polymorphisms and COVID-19-related mortality in Europe. *Journal of molecular medicine (Berlin, Germany)*, *98*(11), 1505–1509. https://doi.org/10.1007/s00109-020-01981-0

Beltz, K., Tsang, D., Wang, J., Rose, S., Bao, Y., Wang, Y., Larkin, K., Rupp, S., Schrepfer, D., Datta, K., Gunderson, K., Sailor, K., Hansen, S., Dobosy, J., Lewis, L., Menezes, A., Walder, J., Behlke, M., Chen, C. (2018). A High-Performing and Cost-Effective SNP Genotyping Method Using rhPCR and Universal Reporters. *Advances in Bioscience and Biotechnology*, 09(09), 497–512. https://doi.org/10.4236/abb.2018.99034

Calabrese, C., Annunziata, A., Coppola, A., Pafundi, P.

C., Guarino, S., Di Spirito, V., Maddaloni, V., Pepe, N., & Fiorentino, G. (2021). ACE Gene I/D Polymorphism and Acute Pulmonary Embolism in COVID19 Pneumonia: A Potential Predisposing Role. *Frontiers in medicine*, *7*, 631148. https://doi.org/10.3389/fmed.2020.631148

Chung, C. M., Wang, R. Y., Fann, C. S., Chen, J. W., Jong, Y. S., Jou, Y. S., Yang, H. C., Kang, C. S., Chen, C. C., Chang, H. C., & Pan, W. H. (2013). Fine-mapping angiotensin-converting enzyme gene: separate QTLs identified for hypertension and for ACE activity. *PloS one*, *8*(3), e56119. https://doi.org/10.1371/journal.pone.0056119

Devaux, C. A., Rolain, J. M., & Raoult, D. (2020). ACE2 receptor polymorphism: Susceptibility to SARS-CoV-2, hypertension, multi-organ failure, and COVID-19 disease outcome. *Journal of microbiology, immunology, and infection = Wei mian yu gan ran za zhi*, *53*(3), 425–435. https://doi.org/10.1016/j.jmii.2020.04.015

Faustine, I., Malik, A., Andrajati, R., & Wanandi, S. I. (2021). Clinical Characteristics and Severity Profile of COVID-19 Patient with Hypertension in Palu, Central Sulawesi. *Indonesian Journal of Pharmacy*, *32*(4), 563-572. https://doi.org/10.22146/ijp.2729

Faustine, I. (n.d). Distribution of ACE gene InDel genotypes and clinical characteristics of patients with hypertension and COVID-19 in Palu City and Lahat District, Indonesia. *Unpublished*.

Glenn, K. L., Du, Z. Q., Eisenmann, J. C., & Rothschild, M. F. (2009). An alternative method for genotyping of the ACE I/D polymorphism. *Molecular biology reports*, *36*(6), 1305–1310. https://doi.org/10.1007/s11033-008-9313-5

Han, C., Han, X. K., Liu, F. C., & Huang, J. F. (2017). Ethnic differences in the association between angiotensin-converting enzyme gene insertion/deletion polymorphism and peripheral vascular disease: A meta-analysis. *Chronic diseases and translational medicine*, *3*(4), 230–241. https://doi.org/10.1016/j.cdtm.2017.07.002

Heidari, M. M., Hadadzadeh, M., & Fallahzadeh, H. (2019). Development of One-Step Tetra-primer ARMS-PCR for Simultaneous Detection of the Angiotensin Converting Enzyme (ACE) I/D and rs4343 Gene Polymorphisms and the Correlation with CAD Patients. *Avicenna journal of medical biotechnology*, *11*(1), 118–123.

Integrated DNA Technologies Inc (2017). rhAMP™ SNP Assay. Retrieved from https://www.idtdna.com/pages/products/qpcr-and-pcr/genotyping/rhamp-snp-genotyping/rhamp-snp-assays

Jacobs, M., Lahousse, L., Van Eeckhoutte, H. P., Wijnant, S. R. A., Delanghe, J. R., Brusselle, G. G., & Bracke, K. R. (2021). Effect of ACE1 polymorphism rs1799752 on protein levels of ACE2, the SARS-CoV-2 entry receptor, in alveolar lung epithelium. *ERJ open research*, *7*(2), 00940-2020. https://doi.org/10.1183/23120541.00940-2020

Kang, H. J., Kim, C. H., Park, D. S., Choi, S. Y., Lee, D. H., Nam, H. S., Hur, J. G., & Woo, J. H. (2012). The Impacts of ACE Activity according to ACE I/D Polymorphisms on Muscular Functions of People Aged 65. *Annals of rehabilitation medicine*, 36(4), 433–446. https://doi.org/10.5535/arm.2012.36.4.433

Rattanathanawan, K., Thongket, P., Panitcharoen, B., Sukhumsirichat, W. (2018). Studies of single nucleotide polymorphism (SNP) of angiotensin-converting enzyme (ACE) gene and serum ACE protein level in association with hypertension in Thai patient. *J Med Health Sci*, *25*(2), 56–66.

Krishnan, R., Sekar, D., Karunanithy, S., & Subramanium, S. (2016). Association of angiotensin converting enzyme gene insertion/deletion polymorphism with essential hypertension in south Indian population. *Genes & diseases*, *3*(2), 159–163. https://doi.org/10.1016/j.gendis.2016.03.001

Marteka, D., Malik, A., Faustine, I., & Syafhan, N. F. (2022). Clinical profile, treatment, and outcomes of patients with COVID-19 in a tertiary referral hospital in South Sumatera, Indonesia: A retrospective single-center study. *Belitung nursing journal*, *8*(6), 529–537. https://doi.org/10.33546/bnj.2302

Melters, D. P., Bradnam, K. R., Young, H. A., Telis, N., May, M. R., Ruby, J. G., Sebra, R., Peluso, P., Eid, J., Rank, D., Garcia, J. F., DeRisi, J. L., Smith, T., Tobias, C., Ross-Ibarra, J., Korf, I., & Chan, S. W. (2013). Comparative analysis of tandem repeats from hundreds of species reveals unique insights into centromere evolution. *Genome biology*, *14*(1), R10. https://doi.org/10.1186/gb-2013-14-1-r10

Peplonska, B., Adamczyk, J. G., Siewierski, M.,

Safranow, K., Maruszak, A., Sozanski, H., Gajewski, A. K., & Zekanowski, C. (2017). Genetic variants associated with physical and mental characteristics of the elite athletes in the Polish population. *Scandinavian journal of medicine & science in sports*, *27*(8), 788–800. https://doi.org/10.1111/sms.12687

Peta Sebaran | Covid19.go.id (n.d). Retrieved from https://covid19.go.id/peta-sebaran

rs4341 - SNPedia (2021). Retrieved from https://www.snpedia.com/index.php/Rs4341

rs4343 - SNPedia (2021). Retrieved from https://www.snpedia.com/index.php/Rs4343

Sabater Molina, M., Nicolás Rocamora, E., Bendicho, A. I., Vázquez, E. G., Zorio, E., Rodriguez, F. D., Gil Ortuño, C., Rodríguez, A. I., Sánchez-López, A. J., Jara Rubio, R., Moreno-Docón, A., Marcos, P. J., García Pavía, P., Villa, R. B., & Gimeno Blanes, J. R. (2022). Polymorphisms in ACE, ACE2, AGTR1 genes and severity of COVID-19 disease. *PloS one*, 17(2), e0263140. https://doi.org/10.1371/journal.pone.0263140

WHO Coronavirus (COVID-19) Dashboard (no date). Retrieved from https://covid19.who.int/table

Wong, M. K. S. (2016). Angiotensin converting enzymes. in *Handbook of hormones comparative endocrinology for basic and clinical Research*, pp. 263–265. https://doi.org/10.1016/B978-0-12-801028-0.00254-3.