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ORIGINAL

Association between PAI-1 4G/5G polymorphism and COVID-19 patients with different SARS-CoV-2 variants

Asociación entre el polimorfismo PAI-1 4G/5G y los pacientes COVID-19 con diferentes variantes de SARS-CoV-2

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Abstract

Introduction: Previous studies revealed that plasma PAI-1 level increases during SARS-CoV-2 infection and as a result, the probability of coagulation abnormalities during the course of infection increases. To our knowledge, there is no scientific investigation that evaluates the association between PAI-1 4G/5G polymorphism and SARS-CoV-2 variants of concern.
Methods: Total number of 408 individuals were included in the study (204 control and 204 patients). PAI-1 gene 4G/5G (rs1799889 A>G) polymorphism were genotyped by PCR-RFLP. The Ct values of COVID-19 patients were recorded to analyze the association with PAI-1 4G/5G gene polymorphism and viral load.
Results: The PAI-1 4G/4G, 4G/5G, and 5G/5G genotype frequencies were, 24.5%, 40.2% and 35.3% in cases versus 1%, 2% and 97% in controls, respectively. The 4G allele has significant distribution between Delta variant cases and control, but not for the Omicron variant. The distribution of PAI-1 4G/5G + 4G/4G and 5G/5G genotype frequencies were, 79% and 21% in the Delta variant versus 51% and 49% in the Omicron variant, respectively. Carriers of the 4G allele had higher viral loads and lower Ct values as well.
Conclusion: Ct values in the distribution of PAI-1 4G/5G + 4G/4G and 5G/5G genotype frequencies were, 21 and 25 in the Delta variant versus 26 and 28 in the Omicron variant.

Key words: PAI-1, polymorphism, COVID-19, SARSCoV-2 VoCs, coagulation.

Resumen

Introducción: Estudios previos revelaron que el nivel plasmático de PAI-1 aumenta durante la infección por SARS-CoV-2 y, como resultado, aumenta la probabilidad de anomalías de la coagulación durante el curso de la infección. Hasta donde sabemos, no existe ninguna investigación científica que evalúe la asociación entre el polimorfismo 4G/5G del PAI-1 y las variantes preocupantes del SARS-CoV-2.
Métodos: Se incluyó en el estudio a un total de 408 individuos (204 controles y 204 pacientes). El polimorfismo 4G/5G (rs1799889 A>G) del gen PAI-1 se genotipificó mediante PCR-RFLP. Se registraron los valores Ct de los pacientes COVID-19 para analizar la asociación con el polimorfismo 4G/5G del gen PAI-1 y la carga viral.
Resultados: Las frecuencias de los genotipos PAI-1 4G/4G, 4G/5G y 5G/5G fueron, 24,5%, 40,2% y 35,3% en los casos frente a 1%, 2% y 97% en los controles, respectivamente. El alelo 4G tiene una distribución significativa entre los casos de la variante Delta y el control, pero no para la variante Omicron. La distribución de las frecuencias de los genotipos PAI-1 4G/5G + 4G/4G y 5G/5G fueron, respectivamente, 79% y 21% en la variante Delta frente a 51% y 49% en la variante Omicron. Los portadores del alelo 4G también presentaron cargas virales más elevadas y valores Ct más bajos.
Conclusiones: Los valores de Ct en la distribución de frecuencias de genotipos PAI-1 4G/5G + 4G/4G y 5G/5G fueron, 21 y 25 en la variante Delta frente a 26 y 28 en la variante Omicron.

Palabras clave: PAI-1, polimorfismo, COVID-19, SARSCoV-2 VoCs, coagulación.

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Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is the RNA virus causing the COVID-19 pandemic, and it has a high mutation rate. So far, five different variants have been deemed variants of concern (VOCs) by the World Health Organization due to increased transmissibility, morbidity and, decreased susceptibility to antiviral drugs and neutralizing antibodies, and ability to evade natural immunity and infect vaccinated individuals[1]. These VOCs are Alpha (B.1.1.7, United Kingdom), Beta (B.1.351 South Africa), Gamma (P.1, Brazil), Delta (B.1.617.2, India), and Omicron (B.1.1.529, South Africa). According to the data which were published by Near East University DESAM Research Institute, the SARS-CoV-2 Omicron variant has been the most rampant variant after Delta variant in Northern Cyprus since the 17th of December 2021 when the first case was seen in Northern Cyprus.

A wide range of symptoms (from mild to severe) was reported for patients with exposure to COVID-19. While most infected individuals have had mild symptoms and recovered without any treatment, some cases were showed a variety of severe phenotypes with multiple symptoms, such as fever, tiredness, cough and loss of taste or smell. Recent published case data suggested that out of all hospitalized patients, 20-30% of them suffer from endothelial injury caused by coagulation and 10% of the hospitalized patients have thrombotic complications[12;14]. Additionally, it is observed that 79% of the patients who have died from COVID-19 developed pulmonary artery thrombosis with coagulation and thrombosis being the main forces behind endothelial cell injury[2;17].

The mechanism of infect to humans of SARS-CoV-2; it binds the host cellular angiotensin-converting enzyme-2 (ACE2) receptor, which the virus has strong binding affinity, and entries the host cell, and replicate its viral genome[2;21]. ACE2 is an angiotensin-converting enzyme, and the virus halts the production of angiotensin when it binds to the ACE2 receptor. Without angiotensin, antithrombotic and anti-inflammatory activities in the endothelial cells decrease, thus coagulation and thrombosis could be observed in some of the hospitalized COVID-19 patients[2]. In hospitalized COVID-19 patients, coagulation markers are regularly observed and antithrombotic drugs such as Clexane or Enoxaparin are administered if the need arises[9]. Thrombosis and coagulation are also symptoms of a weakened fibrinolytic activity, and it can be described as the result of multiple plasminogen inhibitors' interaction with multiple plasminogen activators[3;7]. Transformation of plasmin into plasminogen is achieved by plasminogen activators[8]. Plasminogen is mainly synthesized by the liver and can be found in the plasma and its activation from plasmin is mediated by tissue-type (t-PA) and urokinase-type (u-PA) plasminogen activators. Both mentioned activators are tightly regulated by plasminogen activator inhibitors (PAIs), but the main inhibitor of fibrinolysis is *PAI-1*[6]. Increased *in vivo* expression of PAI-1 decreases the fibrinolytic activity and decreased fibrinolytic activity could lead to thrombosis and coagulation in COVID-19 infected hospitalized patients[7]. Furthermore, higher plasma levels of PAI-1 also are linked with increased clot lysis time and it could be an indicator of COVID-19 severity in hospitalized patients in order to prevent poor end results[4;10;22].

The 4G/5G polymorphism is the single guanosine insertion/deletion variation (5G or 4G) in the PAI-1 gene promoter region, situated 675 base pairs upstream from the transcriptional start site. And the transcription factors have access to a supplemental binding site in the 5G allele, while is absent in the 4G allele[18]. Furthermore, plasma PAI-1 levels are genetically determined to a certain degree[7]. A study in Western populations showed that, 5G homozygotes have the lowest PAI-1 concentrations, while 4G homozygotes have the highest PAI-1 concentrations[7]. A recent study showed that a link between PAI-1 4G/5G polymorphism and COVID-19 and they reported that 4G homozygote patients have the highest plasma PAI-1 concentrations followed by 4G/5G heterozygotes and the least amount of plasma concentration was found in patients with 5G homozygote also the polymorphism associated with severity of the disease[19]. Abdullaev and colleagues also support previous findings, and they have observed that of all COVID-19 patients identified with PAI-1 4G/5G polymorphism have showed thrombotic events[1]. The postmortem examinations have also done in the same study and they revealed that the thrombotic events were mainly raised from pulmonary artery thrombosis or pulmonary embolism with deep vein thrombosis[1]. In addition, the ratio of women who have suffered from pulmonary embolism was twice as much compared to men[1].

Furthermore, Behling et al. have reported a four-day-old fetal autopsy that has shown signs of placental injury caused by coagulation alteration due to fetal SARS-CoV-2 infection, which the mother who had infected in the last trimester of pregnancy and she had homozygote in the 4G allele[11]. They conclude that SARS-CoV-2 passed the placental barrier from the mother who is carried the *PAI-1* 4G/5G polymorphism to infect the fetus and the polymorphism could increase the damage of the SARS-CoV-2 infection[11].

Even though there have been a few studies about the interaction between the *PAI-1* 4G/5G polymorphism and SARS-CoV-2, the effects of different SARS-CoV-2 variants of concern on the individuals with this polymorphism have yet to be researched. Thus, this study aimed to investigate the association between SARS-COV-2 Delta and Omicron (B.1) variants, viral loads and the *PAI-1* 4G/5G polymorphism.

Materials and methods

Sample Collection: A total number of 408 individuals who admitted to Near East University Hospital COVID-19

PCR Diagnosis Laboratory for routine SARS-CoV-2 RT-PCR test were used in this study. The control group consisted of 204 individuals who were SARS-CoV-2 RT-qPCR negative. On the other hand, the case group consisted of 204 patients whom 100 of them were SARS-CoV-2 RT-qPCR positive, infected with SARS-CoV-2 Delta variant and 104 of them were SARS-CoV-2 RT-qPCR positive, infected with SARS-CoV-2 Omicron (BA.1) variant.

Detection of SARS-CoV-2 and identification of VoCs: The 204 patients, who detected of SARS-CoV-2 with Real-Time Polymerase Chain Reaction (RT-qPCR) approach using UNIPLEX SARS-CoV-2 RT-qPCR Detection Kit (Nicosia, Northern Cyprus), included to variants identification analysis by the use of the Multiplex SARS-CoV-2 VoC RT-qPCR detection kit (Nicosia, Northern Cyprus). The samples were analyzed for H69-70 deletion, N501Y, K417N, T478K, Y144del, and P681R mutations for segregation of the VoCs of SARS-CoV-2 between Delta (B.1.617.2) and Omicron (BA.1).

The segregation was done as follow:

- Delta variant: the sample was negative for the mutation H69-70 deletion, N501Y, K417N, Y144del mutations and positive for the Spike T478K and P681R mutations.
- Omicron variant: the sample was negative for the P681R mutation and positive for the H69-70 deletion, N501Y, T478K, K417N, and Y144del mutations.

The whole-genome sequencing analysis was done for confirmations of the VoCs denotations of samples (GISAID reference numbers EPI_ISL_12574367, EPI_ISL_12574374, EPI_ISL_12574370, EPI_ISL_12574375,EPI_ISL_12574368,EPI_ISL_12574373, EPI_ISL_12574369, EPI_ISL_12574371, EPI_ISL_12574372, EPI_ISL_12574000).

PAI-1 4G/5G Gene Polymorphism Genotyping: The human genomic DNA was extracted from volunteered SARS-CoV-2 RT-qPCR positive and negative (control) cases using Uniplex RT-qPCR SARS-CoV-2 RT-qPCR Detection Kit (IKAS Medical, Nicosia, Northern Cyprus). *PAI-1* gene polymorphism (rs1799889 A>G) was genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Genotypes were determined according to the presence and absence of the restriction sites that were analyzed and alleles were designated with respect to actual base change according to the Ensemble (http://www.ensembl.org/) genome browser and NCBI SNP database (https://www.ncbi.nlm.nih.gov/SNP/, dbSNP).

Viral load calculation: The cycle threshold (Ct), that is defined the value of the first PCR cycle detected of the viral RNA amplification, is negatively correlated with the viral load. The Ct value of the viral amplification provides to assess semi-quantitatively of the viral load in the host.

Low Ct value represents high level of viral load and high Ct value represents low level of viral load in the host[16;20]. The viral load was grouped according to Ct value as follows:

- 20>Ct value>30 was defined high viral load
- 30>Ct value>35 was defined as low viral load.

Statistical Analysis: Data statistics was done by utilizing SPSS software (Statistical Package for the Social Sciences, SPSS Inc, Chicago, IL, USA, and version 25). Descriptive data and genotype data of the study group were expressed as mean ± standard deviation (SD) or number and frequency, where applicable. Normal and non-normal distributed quantitative variables were differentiated with Student's t-test and Mann–Whitney U test between two groups, respectively. The genotype and allelic frequency distributions of PAI-1 675 4G/5G polymorphisms between the study groups were compared using Chi square (χ^2). Pearson's chi-square test or the Fisher's exact test were used to verify the association of the categorical variables between study groups, when the conditions for using the chi-square test were not verified. Hardy-Weinberg equilibrium (HWE) was assessed by Fischer's exact test. OR and 95 % CI were estimated by binary logistic regression analysis adopting codominant, dominant, recessive and additive inheritance models. Akaike's information criterion (ACI) was utilized in the selection of the most suitable inheritance model for the data available. To assess the differences between groups, the data were log transformed to meet ANOVA criteria and then subjected to one-way ANOVA with Tukey's post-hoc analysis. Relative risks were assessed of PAI-1 675 4G/5G polymorphism in COVID-19 Delta and Omicron (BA.1) variant patients by calculating odds ratios (ORs) and 95% confidence intervals (CIs) that were considered separate outcomes. In all cases differences were considered significant at $p < 0.05$.

Results

General characteristics of the study group

The study group includes 204 COVID-19 patients (100 patients who are infected by the SARS-CoV-2 Delta variant and 104 patients who are infected by the SARS-CoV-2 Omicron (BA.1) variant) and 204 non-infected patients as a control group. The mean age of COVID-19 patient's ± SD was 48.49±11.54 and control group 47.24±12.34 (p=0.290). The gender distribution of the patients' group is 114 (55.9%) female and 90 (44.1%) male for control group 94 (46.1%) female and 110 (53.9%) (p=0.060).

Allelic and genotypic distribution frequency of PAI-1 -675 4G/5G polymorphism in study group.

The genotypic and allelic frequency distributions of PAI-

1 -675 4G/5G polymorphism in COVID-19 patients who are infected by the SARS-CoV-2 Delta and Omicron BA.1variants and the control group are presented in **table I**.

Notable differences were observed in genotype frequencies of *PAI-1* -675 4G/5G polymorphism between SARS-CoV-2 patients and control group (***p=0.001***).

Furthermore, the risk allele of *PAI-1* -675 4G/5G polymorphism was found to be statistically significant (OR=39.05, 95% CI=/18.88-80.78, ***p=0.001***) in SARS-CoV-2 Delta and Omicron BA.1 variants infected patients compared to controls (**Table I**).

The study group includes 204 COVID-19 patients who are infected by the SARS-CoV-2 Delta and Omicron BA.1 variants and 204 non-infected patients as a control group. OR: Odds Ratio, CI: Confidence Interval. χ^2 and HWE tests were used to compare the genotypic and allelic frequency distributions of polymorphisms between the groups. In all cases, differences were considered significant at $p < 0.05$.

Mutation analysis of *PAI-1 -675 4G/5G* polymorphism showed that 64.7% of SARS-CoV-2 positive patients (case group) were carried at least one mutant allele (homozygous or heterozygous), while the control group has consisted of 3% were carried mutation at least one allele. The differences in the distribution of the PAI-1 6754G/5G polymorphism between the two groups were statistically significant (OR=0.17, 95% CI=0.07-0.4, ***p=0.001***) (**Figure 1**).

The distribution of PAI-1 6754G/5G polymorphism in the patients with SARS-CoV-2 Delta and Omicron BA.1 variants

Furthermore, we also investigated of the distribution of PAI-1 6754G/5G polymorphism in SARS-CoV-2 patients who were infected with Delta and Omicron BA.1 variants. We observed that 21% of SARS-CoV-2 Delta variant infected patients had the 5G/5G (wild type) genotype, while 79% of SARS-CoV-2 Delta variant infected patients carried at least one mutant allele (homozygous or heterozygous). However, the 5G/5G genotype was more prevalent in the SARS-CoV-2 patients who were infected with Omicron BA.1 variant (49%), and the frequency of the mutant genotypes (homozygous or heterozygous) was lower (51%) compared to the patients infected with SARS-CoV-2 Delta variant group (OR=3.62, 95% CI=1.95-6.70, ***p=0.001***) (**Figure 2**).

Analysis of PAI-1 675 4G/5G polymorphism based on the four genetic inheritance models in the patients with SARS-CoV-2 Delta and Omicron BA.1 variants

The genotype frequencies were analyzed by four genetic models: additive, co-dominant, dominant, and recessive models in COVID-19 cases. The PAI-1 -675 4G/5G

Table I: The genotypic and allelic frequency distributions of PAI-1 -675 4G/5G SNP in the study group.

SNP	Genotypic Frequencies n (%)			Allelic Frequencies					
Genotype	Cases (n=204)	Control (n=204)	P-Value	Allele	Cases (n=204)	Control (n=204)	χ^2	OR/CI(95%)	P-Value
PAI-1 675-4G/5G									
5G/5G **4G/5G** **4G/4G**	72(35.3) 82(40.2) 50(24.5)	192(97) 4(2) 2(1)	***0.001***	4G/5G	0.55/0.45	0.98/0.02	201.96	39.05/18.88-80.78	***0.001***

Figure 1: *PAI-1 675 4G/5G* polymorphism genotype distribution. 5G/5G (Wild Type), 4G/5G (Heterozygote), 4G/4G (Homozygote).

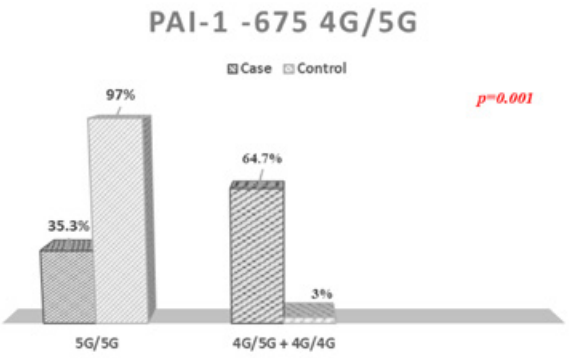
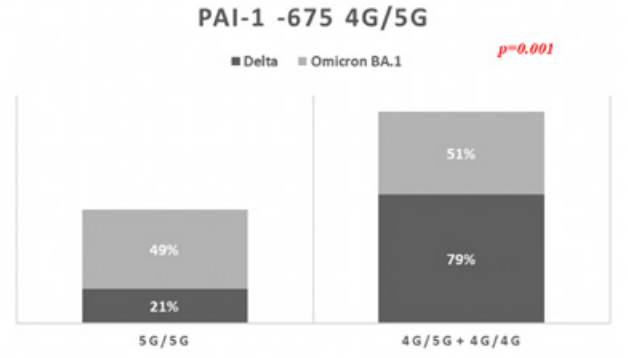


Figure 2: The distribution of PAI-1 6754G/5G polymorphism in the patients with SARS-CoV-2 Delta and Omicron BA.1 variants. *PAI-1* polymorphism genotype distribution 5G/5G (Wild Type), 4G/5G (Heterozygote), 4G/4G (Homozygote).



polymorphism, a significant association between this polymorphism and increased risk of SARS-CoV-2 Delta variant compared to Omicron BA.1 variants cases, and the analysis showed that in all four models, co-dominant genotype (5G/5G) vs (4G/4G) (OR=2.85, 95% CI.=1.34-6.05, p=0.005); co-dominant genotype (4G/4G) vs (5G/5G) (OR=0.35, 95% CI.=0.16-0.74, ***p=0.005***); dominant (OR=3.62, 95% CI.=1.95-6.70, ***p=0.001***); recessive (OR=0.76, 95% CI.=0.40-1.45, ***p=0.417***); additive (OR=0.57, 95% CI=0.11-1.66, ***p=0.002***) (**Table II**).

Associations of the PAI-1 6754G/5G polymorphism and viral load of SARS-CoV-2

To better understand the relationship between *PAI-1 -675 4G/5G* polymorphism and severity of COVID-19, we next evaluated the potential effects of *PAI-1 -675 4G/5G* polymorphism on the viral load of SARSCoV-2. We observed that heterozygote (4G/5G) and homozygote (4G/4G) genotypes carriers of *PAI-1 -675 4G/5G* polymorphism had high viral load in SARS-CoV-2 patients compared to wild type carriers (5G/5G) (*p=0.001*, respectively) (**Figure 3A**).

Also, the low Ct value that means high viral load was found in SARS-CoV-2 Delta and Omicron (BA.1) variants infected patients carried at least one mutant allele carriers (homozygous and heterozygous; 4G/5G+4G/4G) of *PAI-1 -675 4G/5G* polymorphism compared to wild type carriers (5G/5G) (*p=0.001* and *p=0.001*, respectively) (**Figure 3B** and **figure 3C**).

Discussion

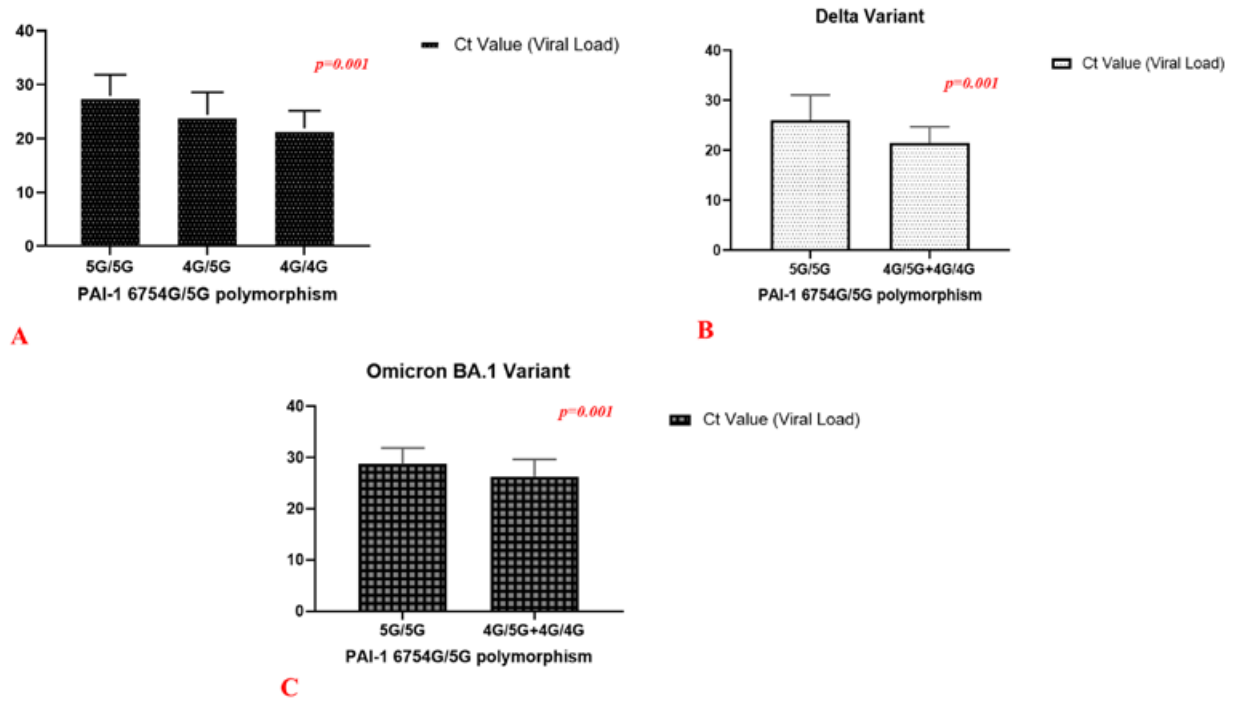
In our study, we have found that the COVID-19 patients who have the 4G allele of the *PAI-1 4G/5G* polymorphism were mainly infected with the Delta variant of the SARS-CoV-2 compared to the Omicron (BA.1) variant.

Table II: Analysis of SNPs based on the four genetic inheritance models.

SNP	Model of Inheritance	OR (95 % CI)	p-Value	AIC[a]
PAI-1 -675 4G/5G	**Co-dominant** 5G/5G vs 4G/4G 4G/4G vs 5G/5G	 2.85 (1.34-6.05) 0.35 (0.16-0.74)	 0.005 0.005	-
	Dominant 5G/5G vs 4G/5G+4G/4G	3.62 (1.95-6.70)	0.001	13.15
	Recessive 4G/4G vs 4G/5G +5G/5G	0.76 (0.40-1.45)	0.417	15.39
	Additive 4G/4G vs 4G/5G vs 5G/5G	0.57 (0.11-1.66)	0.002	16.32

The AIC: the preferred inheritance model is the one with the minimum AIC value. OR; Odds ratio, CI; Confidence interval, AIC; Akaike's information criterion. p-value≤0.05 considered statistically significant. p-values in bold remained significant after Bonferroni correction.

Figures 3 A, B, C:



Our study associated how many COVID-19 patients have which type of polymorphism with respect to whether they are infected with the Delta or the Omicron variant. One observation we made is that 4G homozygous and 4G/5G heterozygous individuals contracted the Delta variant more and 5G homozygous people contracted the Omicron variant more. A study which was done before the emergence of the Omicron and at the time when the Delta variant was the most dominant strain on the planet reported that COVID-19 patients with the 4G allele make up the most populated patient group of them all; a result that is in line with our findings[19].

These finding weren't the first time that *PAI-1 4G/5G* polymorphism has been correlated with a susceptibility increasing effect on certain diseases. Although the disease in question wasn't COVID-19, a study has found out that individuals who have the *PAI-1 4G/5G* polymorphism are more susceptible to T2DM which further supports our findings and the possibility of PAI-1 4G/5G polymorphism makes people vulnerable to COVID-19[15].

Two separate studies have associated the 4G allele with the thrombotic complications caused by coagulation alterations in severe COVID-19 patients and their findings supports ours in which the 4G allele could be used as an indicator of a unexpectedly more severe course of SARS-CoV-2 infection; especially if the patient was infected with the Delta variant of concern[1;11].

If a COVID-19 patient is infected by the Delta variant of SARS-COV-2, then the infection fares more severe compared to a COVID-19 infection caused by the Omicron (BA.1) variant. In terms of Delta variant caused COVID-19, our findings have shown that 4G homozygous patients show the lowest Ct values followed by heterozygote and 5G homozygote patients respectively. A recent study have shown the correlation between lower Ct values with increased chance of worse outcomes and this association further strengthens the idea of that the people who have the 4G allele are more susceptible to COVID-19 infection as well as the course of infection is more severe that those who are 5G homozygous[5]. Thus, both the viral load and the variant of infection could be utilized as an COVID-19 severity indicator.

Conclusion

Our study has found a significant association between the 4G allele of *PAI-1 4G/5G* polymorphism and increased susceptibility to the Delta variant of SARS-CoV-2 whereas, the same association wasn't found with the Omicron variant. Our findings revealed a similar pattern in patients' Ct values as well. Patients who have the 4G allele show lower Ct values, meaning more severe infections, if they were infected by the Delta variant of concern rather than the Omicron (BA.1) variant. Thus, the 4G allele of this polymorphism has potential to be a predictor of infection severity in COVID-19 Delta variant infections.

Ethical approval

All procedures performed in this study were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards (Approval number: YDU/2022/99-1486).

Conflict of Interest

The authors do not have any conflict of interest to declare.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Author Contribution

Conceived and designed the analysis: E.M., G.A., M.C.E.; Collected the data: E.M., G.A., A.T., G.T., E.U.E., H.E., K.S., M.C.E; Contributed data or analysis tools: E.M., G.A., A.T., G.T., E.U.E., H.E., K.S., M.C.E ; Performed the analysis: E.M., A.T.; Wrote the paper: E.M., G.A., M.C.E.; revised the paper: E.M., G.A., A.T., G.T., E.U.E., H.E., K.S., T.S., M.C.E; supervised the project: M.C.E

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