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Article

Genotyping of rs228666 SNP of the human gene ACE2 in mild, moderate and sever Covid-19 patients.

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Abstract: The *ACE2*-converting enzyme has been identified as the specific receptor for Corona virus, but the effect of the *ACE2* gene polymorphism is still unknown, so the expression of human *ACE2* independently may affect the susceptibility to infection with Covid-19. Corona virus is an infectious and pandemic disease caused by it. To evaluate the association between the *ACE2* gene polymorphism and the severity of Covid-19 infection, we used PCR polymerase reaction and polymorphism methods to examine 80 patients classified into severe infection based on symptoms and 80 control factors.. Results demonstrated statistical significance in this high-risk group and the function of rs228666 SNP in the risk of infection with the independent *ACE2* gene rs228666. Individuals who have the variation A allele may be more vulnerable to infection than those who carry G in a condition with the GA genotype when compared to other genotypes GG, GA, while the AA genotype was not significant.Corona virus susceptibility and disease severity were related to inflammation and genetic polymorphism, while there was no clear evidence linking this rs228666 SNP to the severity of Covid-19 infection. At the 0.01 % probability level, the prediction of the GG genotype for control was not significant. In the case of mild infections, the *ACE2* rs228666 SNP genotype was found to be a protective factor for infection with Covid-19 illness in Iraqi patients. Furthermore, patients with this GA genotype are more likely to develop Covid-19 illness than the other genotypes, although the AA genotype had no significant effect on this disease. Individuals with the A allele are also more likely to be infected with COVID-19 than those with the G allele, according to the findings. The GG genotype of the *ACE2* rs228666 SNP was substantially greater at the 0.01 % probability level in the medium cases compared to the control group, showing that the GG genotype of the *ACE2* rs228666 SNP is a protective factor against the incidence of infection. With Covid-19 in Iraqi patients, however the values of the AA genotype are not significant, implying that an individual who carries the A allele is more likely to get Covid-19 disease than those who possess the GG allele.

Keywords: Coronavirus , SNP , *ACE2*.

1. Introduction

Coronaviruses (CoVs) are enveloped viruses containing positive-sense single-stranded RNA as their genetic material[1,2] .The phylogenetic analyses of its genome indicated SARS-CoV-2 as a member of the genus *Betacoronavirus*, which also includes SARS-CoV, SARSr-CoV, MERS-CoV and many other viruses

as well reported to have been isolated from humans and other animal species[3] .Although SARS-CoV-2 shares more than 93.1% sequence similarity of spike (S) gene with of BatCoV RaTG13, the similarity percentage with SARS-CoV and other SARSr-CoVs has been exhibited to be lower than[4] 80% . Paradoxically, although *ACE2* receptor provides the entry point for some coronaviruses into the body, it has been found to play a protective role in several pathophysiological processes too. These include alleviating pathological changes in acute lung injury and acute respiratory distress syndrome[5] , protection against diabetes, hypertension, cardiovascular disease and organ damage as well, by regulation of the renin-angiotensin system (RAS)[6] .The RAS maintains the blood pressure homeostasis in addition to fluids and salts balance which is crucial for the pathological and physiological regulation of several organs including kidney, heart and lungs[7]. *ACE2* primarily functions by negatively regulating the RAS[8] and via degrading and converting the Angiotensin II (potent in vasoconstriction, pro-fibrosis and pro-inflammation) to Angiotensin 1-7 (vasodialatic, apoptotic and proliferative)[5].

2.Materials and Methods

The RealLine Extraction 100 kit can be applied in clinical practice during diagnostics of different human infections and diseases by the polymerase chain reaction (PCR) method and by reverse transcription with subsequent polymerase chain reaction (RT-PCR).Extraction of Human DNA from Whole Blood Total genomic DNA isolated from the whole fresh blood have been collected in EDTA containing tubes for molecular studies, was applied using genomic DNA purification kits (The WizPrep™ DNA Extraction Kit).

Primer preparation:

Table (3-9): Sequences of primer ***ACE*-2 SNP rs 228666.**

Name of primers	Sequence of primers	Reference
ACE2 IF	5'-CATAATCACTACTAAAAATTAGTATCC-3'	Newly Designed
ACE2 IR	5'- TTATTACTTGAACCAGGGAA -3'	
ACE2 OF	AAGTAAATGTGATACAATTTACAAG	Newly Designed
ACE2 OR	AAAGGATATCTTTATATTAGCATTC	
Product For A allele	159	
Product for G allele	200	
Product for two outer	313	

*IF: Inner Forward OR: Outré Reverse

Temperatures and time of T-ARMS PCR Programs:

Optimization of PCR reaction was accomplished after several trials for annealing phase (Annealing 51C^{o}) .It was then selected temperature of 51C^{o} that give the best results for polymerase chain reactions. Been dealing with other temperatures (denaturation and Extension) without changing the previous research program for PCR (*ACE-2*) gene (thus the following programs were adopted .The PCR reaction was carried out as shown in Table(3-10).

Table (3-10): PCR Programs for ***ACE*-2 SNP rs 228666** of T-ARMS.

Steps	Temperature (°C)	Time (minutes)	No. of Cycles
Initial denaturation	96	2	1
First loop:			
Denaturation	96	1	30
Annealing	51	1	
Extension	72	1	
Final extension	72	10	1

3.Results and Discussion

The results of genotypes and alleles frequencies of rs2286666 SNP in *ACE2* gene in controls and patients with Covid-19 are presented in Tables 4-1, 4-2, 4-3 and 4-4. The percentage of wild-type GG genotype was non significantly ($p \leq 0.01$) slightly lower in mild Covid-19 patients group than in controls group (87.5% *versus* 77.5%, respectively) which represent that genotype GG of the *ACE2* rs2286666 a protective factor against the incidence of Covid-19 in Iraqi patients (OR:1.013) (Table 4-1, Figure 4-1). The results also showed that the percentage of heterozygous mutant GA genotype of the SNP was significantly ($p \leq 0.01$) higher in mild Covid-19 patients than in controls (6,25% *versus* 12%, respectively) which represent that patients with this genotype could be in risk to have Covid-19 disease than other genotypes (OR: 1.4384). While mutant genotype AA result showed a non-significant effect on disease infection. The present results showed that individual with the variant A allele 24 (30%) may have a risk for Covid-19 disease than those carrying G homozygote 136 (70%).

The results of genotypes and allele frequencies of rs228666 SNP of the *ACE* 2 gene in controls *versus* moderate Covid-19 patients represent in Table 4-2, Figure 4-2 and revealed that the wild type GG genotype was significantly ($p \leq 0.01$) high lower in moderate Covid-19 patients group than in controls group (87.5% *versus* 72.5%, respectively) which indicate again that genotype GG of the *ACE2* rs2286666 is a protective factor against the incidence of Covid-19 in Iraqi patients (OR: 1.037).

On the other hand, heterozygous mutant GA genotype of the SNP was significantly ($p \leq 0.01$) higher in moderate Covid-19 patients than in controls (6,25% *versus* 18.75%, respectively, OR:3,571) which represent that patients with this genotype could be in risk to have a Covid-19 disease than other genotypes. While mutant genotype AA result was non-significant (6.25% *versus* 8.75%, respectively) with OR, 0.967. These

results showed that individual with the variant A allele 29 (18%) could be in risk for Covid-19 disease than those carrying G homozygote 131 (82%).

observed[9] that severely affected COVID-19 infection is related to the virus's expression of *ACE2*, and it was found that the low level of infection with this virus in terms of infection and complications is mild because levels of *ACE2* in the serum are higher in children than in adults, which is the first point of contact, as most epidemiological data showed that the majority of COVID-19 cases in children showed mild or moderate clinical symptoms[10]. While the aim is to evaluate the correlation between the genetic polymorphisms of *ACE2*, *ACE1*, and their role in determining the course of the disease (mild, moderate, severe), discovering such a relationship determines the severity or severity of the injury, which somewhat corresponds to what was researched. Concerning the role of this gene in determining the severity of infection in our study, because the number of genetic polymorphisms is very important in determining the severity of infection, as shown[11]. the expression levels of the *ACE2* gene play a role in the severity of the disease, which corresponds to what was found in Our study is a case of light injuries, and *ACE2* has been linked to injury severity, whether moderate or mild[12]. which is consistent with the observations. that moderate[13] virus is related to the host's immunological response and is not associated with increased expression of *ACE* receptors. It was also found[14] that people infected with mild cases may have diarrhea or show no symptoms at all, which complicates diagnosis and treatment because infected people may be unaware of the infection and spread it. Also[14] discovered that *ACE2* is one of the key receptors for COVID-19 and that the severity of infection is determined by it. when the *ACE2* gene expression is low, the severity of infection is very low, and this decrease prevents the spread of COVID-19. The infection in these cases is moderate and without symptoms, although the World Health Organization (WHO) reports that these cases exhibit disease signs. While it has been demonstrated[15] that only 33% of average cases develop a fever and 67% do not. Elevated plasma *ACE2* levels in COVID-19 have been found to be substantially linked with disease severity, suggesting that *ACE2* production is involved in virus transmission and the burden of disease[16]. showed that the *ACE2* protein functions as a receptor for virus entry by establishing a special connection between them, and the expression of the *ACE2* protein varies according to organ[17]. It has been discovered[18] that low expression of the *ACE2* protein in the lung is associated with the average condition in the severity of the injury, and all of what has been mentioned gradually agrees with the results obtained despite differences in the type of expression of the *ACE2* protein, whether it is expressed in the lung to accept or reject. Our results were consistent with that of[18,19] who showed that the lower the *ACE2* expression, the more moderate the injury, especially in the lungs, and when the expression of *ACE2* rose in the lungs, In addition to the fact that pulmonary expression of the *ACE2* gene may contribute to the risk of infection with Covid-19, and as many researchers have proposed, the danger of Covid-19 is tied to *ACE2* and its function in viral infection[20].

Table 4-1: Genotype distribution and allele frequency of rs228666 *ACE* 2 gene in COVId-19 mild infected patients and control group.

Genotype (ACE 2)	Healthy No. (%)	Patients No. (%)	Chi-Square (χ^2)	P-value	O.R. (C.I.)
GG-wild	70 (87.5%)	62 (77.5%)	0.457	0.5001*	1.013
GA	5 (6.25%)	12 (15%)	0.3604	0.5501*	1.4384
AA	5 (6.25%)	6 (7.5%)	0.0976	0.7550^{NS}	0.921
Total	80 (100%)	80 (100%)			
Allele	Frequency				
G	145 (90%)	136 (70%)			
A	15 (10%)	24 (30%)			

*(P≤0.05); NS: Non-Significant.

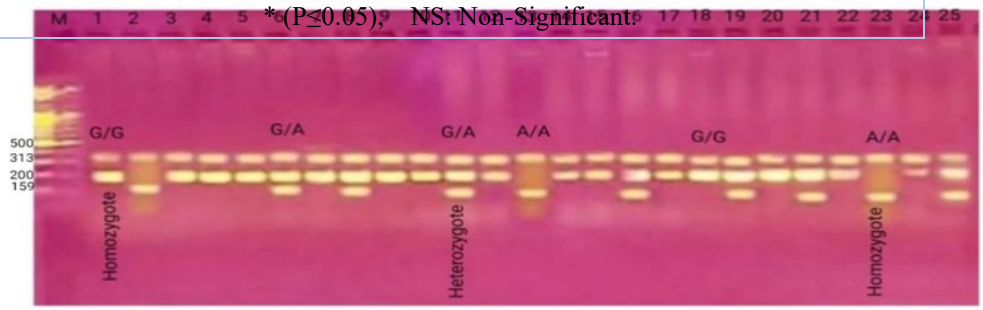

Figure 4-1 :The 200 bp band indicates the G allele and the 159 bp band indicates the A allele. The 313 bp band indicates the product of the outer primers and was used as positive control for the PCR reactions. Genotype GG homozygote (lanes 1,3,4,5,7,9,10,12,14,15,17,18,20,22,24), genotype GA heterozygote (lanes 6,11,19) and genotype AA homozygote (lanes 2,13,23).

Table 4-2: Genotype distribution and allele frequency of rs228666 *ACE* 2 gene in COVId-19Moderate infected patients and control group.

Genotype ACE 2)(	Healthy No. (%)	Patients No. (%)	Chi-Square (χ^2)	P-value	O.R. (C.I.)
GG-wild	**70 (87.5%)**	**58 (72.5%)**	**5.625**	**0.0203***	**1.037**
GA	**5 (6.25%)**	**15 (18.75%)**	**5.714**	**0.0193****	**3.5714**
AA	**5 (6.25%)**	**7 (8.75%)**	**0.3604**	**0.4023 NS**	**0.967**
Total	**80 (100%)**	**80 (100%)**			
Allele	**Frequency**				

G	**145 (90)**	**131 (82%)**		
A	**15 (10%)**	**29 (18%)**		
*** (P≤0.05), ** (P≤0.01), NS: Non-Significant.**				

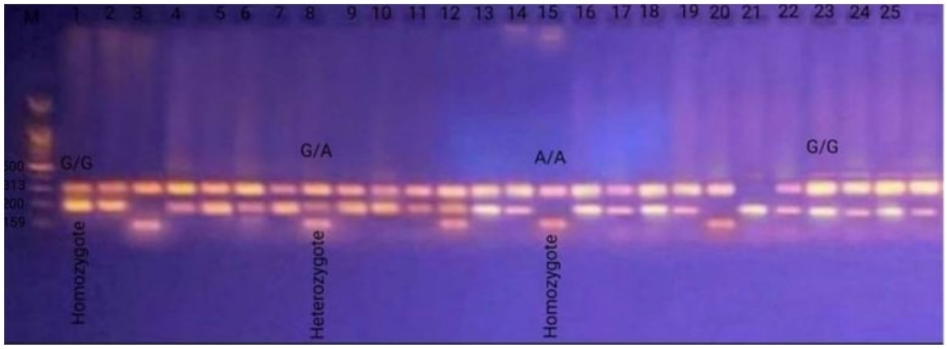

Figure 4-2 :The 200 bp band indicates the G allele and the 159 bp band indicates the A allele. The 313 bp band indicates the product of the outer primers and was used as positive control for the PCR reactions. Genotype GG homozygote (lanes 1,2,5,6,7,9,10,11,13,14,16,17,18,19,22,23,24,25), genotype GA heterozygote (lanes 8,12,) and genotype AA homozygote (lanes 3,15,20).

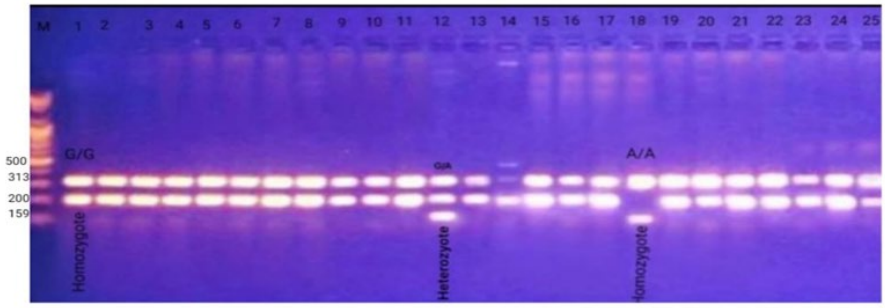

Figure 4-4:The 200 bp band indicates the G allele and the 159 bp band indicates the A allele. The 313 bp band indicates the product of the outer primers and was used as positive control for the PCR reactions. Genotype GG homozygote (lanes 1,2,3,4,5,6,7,8,9,10,11,13,14,15,16,17,19,20,21,22,23,24,25), genotype GA heterozygote (lanes 12) and genotype AA homozygote (lanes 18).

The results of genotypes and allele frequencies of rs228666 SNP of the *ACE* 2 gene in controls *versus* sever Covid-19 patients represent in Table 4-3, Figure 4-3. The results revealed that the wild type GG genotype was significantly (p≤0.01) high lower in sever Covid-19 patients group than in controls group (87.5% *versus* 21.25%, respectively, OR:1.01) which confirm that genotype GG of the *ACE2* rs2286666 is a protective factor against the incidence of Covid-19 in Iraqi patients.

The result of heterozygous mutant GA genotype of the SNP was significantly (p≤0.01) higher in sever Covid-19 patients than in control (6,25% *versus* 70%, respectively, OR:3,571). These result indicated that the severity of patients infected with Covid-19 disease could be due to the genotype of *ACE2* rs2286666 SNP where the severity increased in patients with the genotype GA than other genotypes (GG and GA). On the other hand, mutant genotype AA result was non-significant (6.25% *versus* 8.75%, respectively) with OR, 0.9014. The results also showed that individual with the variant A allele 70 (45%) could be in risk for Covid-19 disease than those carrying G homozygote 90 (55%). Showed[21] that the virus causes an equilibrium by *Ang* 1 in the host cells it targets, which significantly increases lung damage, which is consistent with the

effect on patients in severe cases who suffer from shortness of breath and damage. *ACE2* is a specific receptor for the virus in the lung, although the potential effect of *ACE2* genetic polymorphism is undetermined. Confirmed[22] that this gene affects sensitivity to COVID infection and disease outcome, but there was no effect on infection severity, stating that there is no evidence that the *ACE2* and *ACE1* polymorphisms are directly related to disease severity. linked[23] the severity of infection to differences in individuals based on epigenetic alleles of gene expression of the *ACE2* receptor, which is completely consistent with the observations. While[9] found that the increased susceptibility to Covid-19 infection is related to the expression of the targeted *ACE2* receptor, our results in terms of age in infection with the virus and the role of *ACE2* enzyme coincide with[24] Unlike most other studies that evaluated the relationship between gender and the severity of infection, we did not use gender as an indicator of infection in our current study. These studies concluded that gender really wasn't significantly related to the risk of disease spread, whereas other studies found that men were more susceptible to this disease and more severely[25] and in contrast to what we found, mentioned[26] a negative relationship between *ACE2* and *ACE1* in the severity of infection and mortality, whilst also[27] identified a highly high expression of *ACE2* protein in postmortem lung tissues from Covid-19 patients. What stands out here is the function of the *ACE2* protein in generating infection and infection with COVID 19, as well as patient death. This is consistent with what we discovered about this protein throughout our study.

Table 4-3: Genotype distribution and allele frequency of rs228666 *ACE* 2 gene in COVId-19 sever infected patients and control group.

Genotype ACE 2)(	Healthy No. (%)	Patients No. (%)	Chi-Square (χ^2)	P-value	O.R. (C.I.)
GG-wild	70 (87.5%)	17 (21.25%)	70.77	0.0001 **	1.01
GA	5 (6.25%)	56 (70%)	64.47	0.0001 **	11.247
AA	5 (6.25%)	7 (8.75%)	1.252	0.2694 NS	0.9014
Total	80 (100%)	80 (100%)			
Allele	Frequency				
G	145 (90%)	90 (55%)			
A	15 (10%)	70 (45%)			
* (P≤0.05), ** (P≤0.01), NS: Non-Significant.					

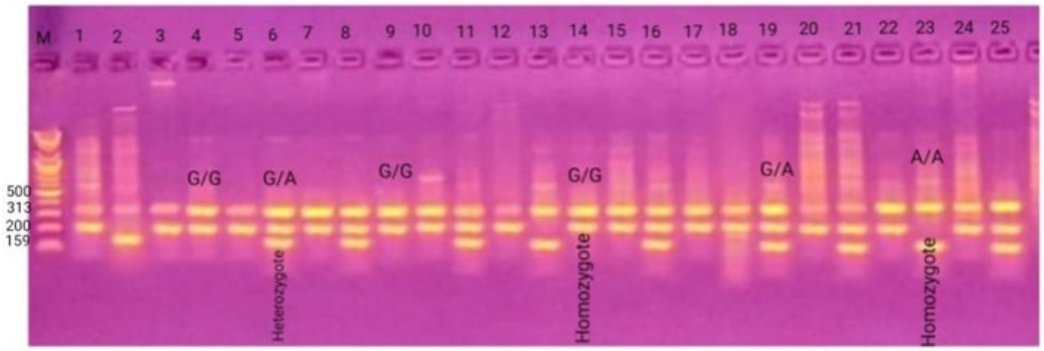

Figure 4-3:The 200 bp band indicates the G allele and the 159 bp band indicates the A allele. The 313 bp band indicates the product of the outer primers and was used as positive control for the PCR reactions. Genotype GG homozygote (lanes1,3,4,5,7,8,10,12,14,15,17,18,20,22,24),genotypeGAheterozygote(lanes6,8,11,16,19,21,25) and genotype AA homozygote (lanes 2,13,23). .

4. Conclusions

Genotype GG of the *ACE2* rs228666 is a protective factor against the incidence of Covid-19 in Iraqi patients. Heterozygous mutant GA genotype of the SNP *ACE2* rs228666 could be play a role in risk to have a Covid-19 disease than other genotypes.

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