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Research Article

Transmembrane Serine Protease-2 Gene Polymorphism and Expression in Iraqi COVID-19 Patients

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A B S T R A C T

Introduction: The global COVID-19 pandemic was caused by SARS-CoV-2. Human cells ingest this virus when ACE2 identifies it. *TMPRSS2* prepares SARS-CoV-2 for entry. The clinical outcomes of COVID-19 are associated with ACE2 and *TMPRSS2* gene expression polymorphisms.

Objective: To determine if the *TMPRSS2* gene rs 2070788 SNP in intron 11–12 is associated with severe COVID-19 in Iraqi patients

Methods: The study included 120 COVID-19 patients from three Ramadi City hospitals and 80 healthy controls. DNA extraction was done with Wizard genomic TM DNA Extraction Kit, RNA extraction was done with One Script Plus cDNA Synthesis Kit, and qRT-PCR was used for investigating genetic polymorphism.

Results: The *TMPRSS2* rs2070788 SNP had three genotypes (CC, CT, and TT) and two alleles. The genotypes of COVID-19 patients and controls were compatible with Hardy-Weinberg equilibrium (HWE). Severe COVID-19 patients and controls had significantly higher TT genotype frequencies in *TMPRSS2* (28% vs 4%, OR = 9.33; 95% CI = 2.03 to 43.01; p = 0.002) and T allele (48% vs 16%, OR = 2.37; 95% CI = 1.32 to 4.26; p = 0.005), respectively. *TMPRSS2* mRNA expression was much lower in severe cases than controls. In addition, the relative expression of *TMPRSS2* mRNA was increased by 1.15 ± 0.71 folds in the CC genotype of rs2070788 SNP compared to the CT (0.632 ± 0.25) and TT (0.552 ± 0.193) genotypes, but the difference was not significant (p > 0.05).

Conclusion: Iraqis were highly susceptible to COVID-19 due to the rs2070788 TT genotype and T allele. Severe cases also downregulated *TMPRSS2* gene expression.

Keywords: COVID-19, *TMPRSS2*, Gene Polymorphism, Gene Expression, RT-PCR

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Introduction

Coronavirus disease-2019 (COVID-19) is caused by a new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which was initially found in Wuhan, China, in late December 2019. A variety of viruses such as HCoV-229E, SARS-CoV, and MERS, including SARS-CoV-2 employ the transmembrane serine protease 2 (*TMPRSS2*) as a primary host protease to penetrate human cells. This is found in high concentrations in specific parts including lung tissue and bronchial transient secretory cells.[1] The angiotensin-converting enzyme 2 (ACE2) has been described as the entry receptor for SARS-CoV-2 and the *TMPRSS2* is an important priming enzyme required during this process.[2] It cleaves the viral spike (S) protein into S1 and S2 to facilitate the fusion of the virus with cellular membranes.[3] The co-expression of ACE2 and *TMPRSS2* genes has been detected by single-cell RNA-sequencing analyses on nasal mucosa cells, type-2 pneumocytes in lungs, and absorptive enterocytes in small intestine, characterizing potential initial target sites for SARS-CoV-2 replication in humans.[4] However, data from ACE2 and *TMPRSS2* expression on different respiratory tract sites of SARS-CoV-2 infected individuals are still incomplete and have been raising divergent hypotheses about their potential impact on COVID-19 susceptibility and disease severity.[5] The *TMPRSS2* encoding gene is located on chromosome 21 (21q22.3) and contains 15 exons and a reading frame for 492 amino acids.[6] This enzyme contains three main regions: its N-terminal region is located inside the cytoplasm, and next to it, there is an LDL receptor region that binds to calcium, and finally, there is a third region that is rich in the amino acid cysteine (SRCR) which binds to other extracellular molecules. Its extracellular region is a catalytic region, activated by the autocatalytic process, entering the extracellular space and separating from the rest of the enzyme.[7] This enzyme has two isoforms, due to the alternative mRNA splicing process. Isoform 2 contains 492 amino acids, and isoform 1 contains 37 additional amino acid residues in the N-terminal region of this protein.[8] It has been shown that some variants of the *TMPRSS2* gene can affect the severity of viral diseases such as influenza and COVID-19.[9] Given the relevance of the *TMPRSS2* gene in the SARS-CoV-2 infection process, COVID-19 infection and severity pattern may be directly linked to elevated *TMPRSS2* gene polymorphism and expression, resulting in varying disease susceptibility outcomes in various communities. However, the role of *TMPRSS2* polymorphism and expression in disease susceptibility in the Iraqi populations is largely unexplored. Therefore, this study aims to examine the potential correlation between *TMPRSS2* gene rs 2070788 SNP located in the intron 11–12 and its expression in severe COVID-19 among Iraqi patients.

Material and Methods

Patients and Controls

A case-control study was conducted, involving 120 patients who tested positive for COVID-19 and were admitted to three hospitals in Ramadi (Al-Shiafa Hospital, Al-Anbar General Hospital, and Women Children Hospital) for diagnosis and treatment between December 2020 and June 2021. Additionally, 80 individuals who were apparently healthy were included as controls. The individuals were confirmed to have COVID-19 through molecular testing using nasopharyngeal swabs and a chest computed tomography (CT) scan. The apparently healthy controls were investigated for nasopharyngeal swabs, ESR and CRP and the results of these tests were negative. The patient group was found to have severe symptoms according to the World Health Organization's (WHO) Interim Guidance.[10] Blood specimens of 2 ml, drawn from the study participants, were transferred to EDTA tubes and frozen at -20 ºC for DNA extraction, while one nasopharyngeal swab was placed in one PCR tube each containing triazole for RNA extraction.

Molecular Study

DNA Extraction and Preparation

Wizard® Genomic DNA Purification Kit (Promega, USA) was utilised to extract DNA from blood specimens and triazole reagent (Thermo Scientific, USA) was used to extract RNA from nasopharyngeal swabs. Quantus Fluorometer (Promega, USA) was employed to measure the DNA and RNA concentrations.

Detection of *TMPRSS2* rs2070788 Gene Polymorphism

Conventional PCR (Applied Bio-system USA) was employed to assess the polymorphism of the *TMPRSS2* gene at a position of 988. PCR was done with a total volume of 25 µl in a PCR tube. The tube contained 12.5 µl OneTaq 2X Master Mix with standard buffer (Promega, USA), 5 µl DNA template, 1.5 µl of each of the designed reverse primer (GTTTGTCTCCCATCTGTCTG) and sequence-specific forward primer (GAACAGCTCTGCTGGATTT), and 4.5 µl of nuclease-free water. Then in a microcentrifuge, the reactions were centrifugated for 5 seconds and placed in a thermal cycler. After several trials, the thermocycling conditions for amplification were 30 seconds at 94 °C, 30 cycles of 30 seconds at 94 °C, 30 seconds at 49 °C, 1 min at 72 °C, and 5 min at 72 °C. PCR products were visualised by 1% agarose gel electrophoresis stained with Red Safe solution. Then, PCR fragments that were amplified were sequenced using Sanger's method with the ABI3730XL automated DNA sequencer (Macrogen Corporation, Korea).

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Detection of *TMPRSS2* Gene Expression

OneScript® Plus cDNA Synthesis Kit (ABM, Canada) contains all materials required for first-strand cDNA synthesis using random primers. The procedure was performed at a reaction volume of 20 µl (1 µl OneScript® Plus Reverse Transcriptase, 4 µl RT buffer, 1 µl dNTP, 1 µl random primers, 10 µl extracted RNA, and 3 µl of nuclease-free H_2O) according to the manufacturer's instructions. The reaction components were gently mixed and briefly centrifuged. Then the reaction tubes were loaded into the thermal cycler after programming for the following conditions: annealing at 25 ºC for 10 mins, DNA polymerization at 37 ºC for 120 mins, enzyme deactivation at 80 ºC for 5 mins and holding at 4 ºC.[11] The first-strand cDNA can be directly used as a template in PCR. Luna® Universal qPCR Master Mix (BioLabs, New England) was used to assess the expression of the *TMPRSS2* gene. The reaction mixture with a final volume of 20 µl (10 µl qPCR Master Mix, 1 µl forward primer, 1 µl reverse primer as shown in Table 1, 3 µl cDNA template, and 5 µl nuclease-free water) was prepared on q PCR tubes. There were two PCR tubes for each sample, one tube for the *TMPRSS2* gene, and the other for the housekeeping gene (beta-actin (ACTB)).

The qPCR tubes were transferred to the real-time thermocycler, which was programmed for the following optimised cycles (1 cycle for initial denaturation at 95 ºC for 60 seconds and denaturation at -95 ºC for 15 seconds, then 40–45 cycles for extension at 60 ºC for 30 seconds with plate read).

Statistical Analysis

The statistical analysis was performed using the IBM SPSS Statistics 25.0 (Armonk, NY: IBM Corp.). Categorical variables were given as numbers and percentages, and significant differences were assessed using the two-tailed Fisher's exact test. Data of alleles and genotypes of *TMPRSS2* gene SNPs were demonstrated as numbers and/ or percentages. Hardy–Weinberg equilibrium (HWE) was applied to test the differences between study variables and expectations. The odds ratio (OR) and 95% confidence interval (CI) were calculated by logistic regression analysis. A p value of less than 0.05 was considered significant. DNA sequences were analyzed using the Geneious software version 10.2.2 and the significance was accepted when the p value was less than 0.05 using the chi-square test. Data of gene expression were given as mean ± standard error (SEM), and significant differences between means were assessed by ANOVA (Analysis of Variance) followed by either LSD (Least Significant Difference). In both cases, a probability that was equal to or less than 0.05 was considered significant.

Ethical Statement

The ethics committee of the College of Science, University of Anbar approved the present work (Ref. 56 Date 9/6/2022). All the participants gave their informed consent before joining the study.

Results

Demographic Characteristics of Cases and Controls

The age range of the COVID-19 cases was 22–71 years. Most patients were older than 45 years (70.3%), while most healthy subjects were less than 45 years of age (65.0%). Patients and controls were distributed by gender (males and females) while the results did not show significant differences. All studied groups were divided into vaccinated and unvaccinated people. In the control group, most participants were vaccinated (86% vs 14%). However, in severe cases, the unvaccinated were more than the vaccinated (92% vs 8%). The differences were statistically significant ($p < 0.001$) (Table 2).

Table 1.Primers used in TMPRSS2 Gene Expression

S. No.	Primer Name	Description	Sequence 5` ---- 3`	Amplicon Size (bp)
1	ACTB	Forward	GCGAGAAGATGACCCAGA	90
		Reverse	CAGAGGCGTACAGGGATA	
2	TMPRSS2	Forward	CCTCTAACTGGTGTGATGGCGT	121
		Reverse	TGCCAGGACTTCCTCTGAGATG	

Table 2.Demographic Characteristics of COVID-19 Cases and Controls

Characteristics		Cases (N = 120)	Control (N = 80)
Age groups (years)	< 45	29.7%	65.0%
	≥ 45	70.3%	35.0%
	p value	< 0.001	< 0.001
Gender	Male	65.2%	64.0%
	Female	34.8%	36.0%
	p value	> 0.05	> 0.05
Vaccination status	Vaccinated	8.0%	86.0%
	Unvaccinated	92.0%	14.0%
	p value	< 0.001	< 0.001

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TMPRSS2 Gene Polymorphism

In this section of the present study, *TMPRSS2* rs2070788 SNPs genetic polymorphism was investigated. It is a C > T transition located in intron 11–12 of the *TMPRSS2* gene. Agarose gel electrophoresis of the *TMPRSS2* gene PCR amplified products (rs2070788 SNP) showed a single band of 988 bp molecular size, as shown in Figure 1.

The SNP rs2070788 was presented with three genotypes (CC, CT and TT) and two alleles (C and T) (Figure 2). Analysis of HWE in COVID-19 patients and controls revealed that the genotypes were consistent with the equilibrium, and no significant differences (p > 0.05) were found between the observed and expected genotype frequencies.

Inspecting the *TMPRSS2* genotype and allele frequencies in the group of severe COVID-19 patients and controls revealed a significant increase in TT genotype frequencies (28% vs 4%, OR = 9.33; 95% CI = 2.03 to 43.01; p = 0.002) and T allele frequencies (48% vs 16%, OR = 2.37; 95% CI = 1.32 to 4.26; p = 0.005), respectively (Table 3).

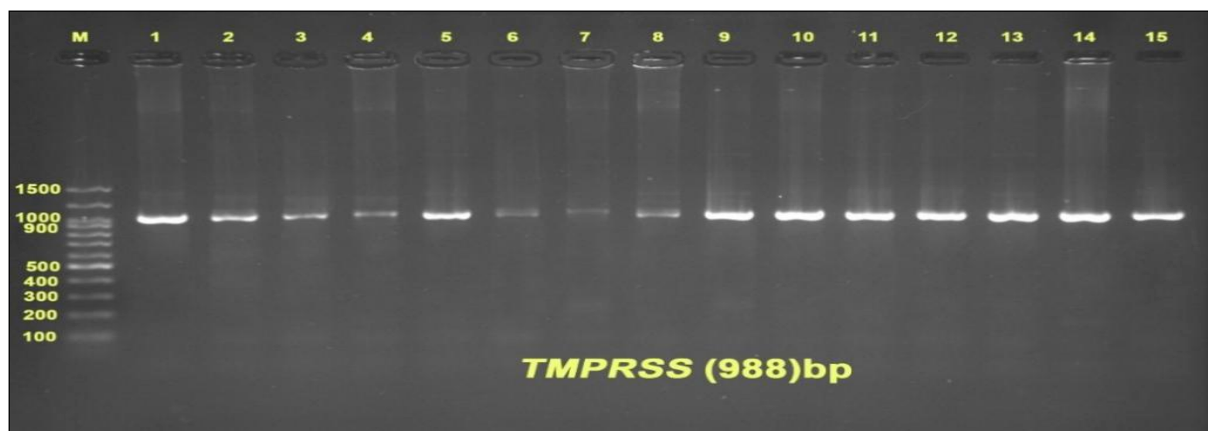

Figure 1.Gel Electrophoresis of *TMPRSS2* Gene PCR Products (SNP rs2070788) on 1% Agarose at 5 V/cm² for 45 Minutes showing Bands of 988 bp Molecular Size (Lane M: 100 bp DNA Ladder; Lanes 1–15: Samples of COVID-19 Patients)

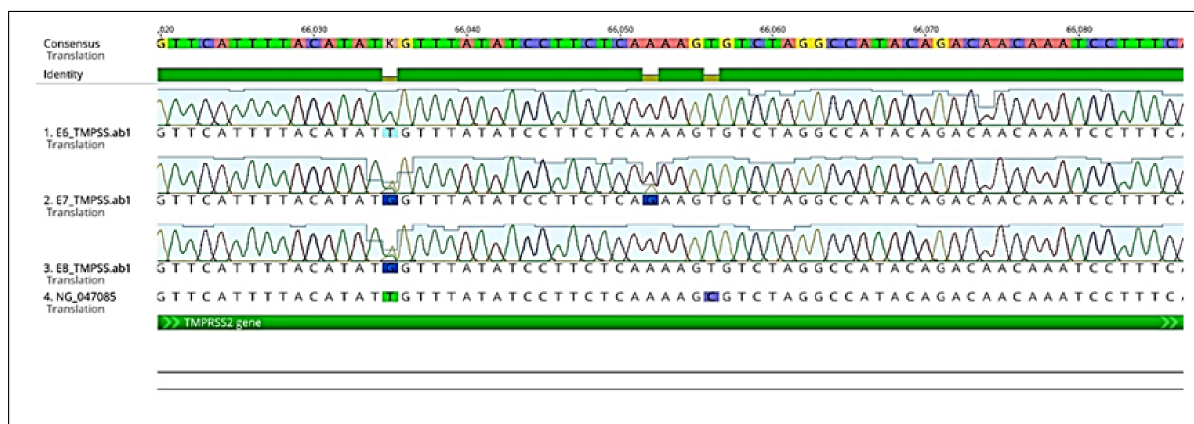

Figure 2.Chromatogram of DNA Sequence regarding *TMPRSS2* Gene SNP (G/A: rs2070788) Revealing 3 Genotypes of CC (Sample 42), CT (Sample 3) and TT (Sample 1) in addition to the reference sequence of rs2070788

Table 3.Statistical Analysis of Association between Genotypes and Alleles of *TMPRSS2* Gene (rs2070788 SNP) and COVID-19

Genotype or Allele	Patients (N = 120)		Controls (N = 80)		Odds Ratio	95% Confidence Interval	p Value
	n	%	n	%			
CC	38	31.7	58	72.50	Reference		
CT	48	40.0	19	23.75	2.11	0.90 to 4.95	0.133 NS

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TT	34	28.3	3	3.75	9.33	2.03 to 43.01	0.002 S
C	124	51.7	125	84.00	Reference		
T	116	48.3	25	16.00	2.37	1.32 to 4.26	0.005 S

p: Probability; NS: Not significant (p > 0.05), S: Significant

TMPRSS2 Gene Expression

The ΔCt mean of the *TMPRSS2* gene was increased in severe COVID-19 patients (11.432 ± 0.39) as compared to the corresponding ΔCt mean in controls (10.039 ± 0.38), and the difference was significant ($p < 0.05$). Furthermore, the relative expression ($2^{-\Delta\Delta Ct}$) of *TMPRSS2* mRNA was significantly less than that of control (0.747 ± 0.206) as compared to the patients (Table 4).

Table 4.Expression of TMPRSS2 mRNA in COVID-19 Patients and Controls

Studied Groups	Num-ber	ΔCt (Mean ± SEM)	p Value	Folding (Mean ± SEM)	p Value
Severe COVID-19 patients	120	11.432 ± 0.39	< 0.05*	0.747 ± 0.206	< 0.05*
Controls	80	10.039 ± 0.38			

*Significant (p < 0.05)

Impact of TMPRSS2 Gene (rs2070788 SNP) on its Expression

To examine the impact of the investigated *TMPRSS2* gene rs2070788 SNP on its expression, the folding means were determined in each genotype of this SNP.

TMPRSS2 Gene (207088 SNP)

The folding mean of *TMPRSS2* mRNA was increased by 1.15 ± 0.71 folds in the CC genotype of rs2070788 SNP as compared to the CT (0.632 ± 0.25) and TT (0.552 ± 0.193) genotypes, but the difference was not significant ($p > 0.05$) (Table 5).

Table 5.Impact of rs2070788 SNP on *TMPRSS2* mRNA Expression in COVID-19 Patients

Genotype	Number	Folding (Mean ± SEM)	p Value
CC	40	1.15 ± 0.71	Not significant (p > 0.05)
CT	54	0.632 ± 0.25	
TT	26	0.552 ± 0.193	

Discussion

The current study assessed the influence of genetic polymorphisms modulating *TMPRSS2* expression on the severity of COVID-19. We determined the frequencies of *TMPRSS2* rs2070788 genotypes and alleles in Iraqi COVID-19 patients. HWE analysis revealed that the genotypes and alleles were consistent with the equilibrium. This result agreed with numerous studies in different populations (German, Brazilian, and Kurdish Iraqi) which reported that transmembrane serine protease 2 rs2070788 genotypes were compatible with HWE.[3,12,13] In the present study, TT genotype and T alleles were found to be associated with severe COVID-19 cases, which is not in accordance with the findings of Schönfelder et al.[3] who pointed out that neither *TMPRSS2* rs2070788 nor rs12329760 polymorphisms were related to SARS-CoV-2 infection risk or severity of COVID-19. Andrade et al.[12] reported that survival analysis demonstrated that older COVID-19 patients carrying the rs2070788 GG genotype had shorter survival times when compared to those with AG or AA genotypes. The rs2070788 polymorphism in *TMPRSS2* was associated with an increased risk of death (four-fold) in older patients hospitalized with COVID-19. A recent Dutch study of 188 adult hospitalized patients demonstrated a protective effect of the rs2070788 AA genotype on COVID-19 severity.[13] Smail et al. pointed out that the GA genotype in *TMPRSS2* rs2070788 SNP was not significantly associated with SARS-CoV-2 infection when compared to the GG genotypes ($p = 0.254$).[14] In contrast, the AA genotype and the A allele were protective for COVID-19 infection ($p = 0.009$ for both). Fernández-de-Las-Peñas et al. found that no differences in long-COVID symptoms were dependent on *TMPRSS2* rs2070788 genotypes.[15] They observed that the G allele of the *TMPRSS2* rs2070788 was associated with higher development of post-COVID dyspnoea and gastrointestinal disorders. The differences in previous results may be attributed to the small sample size in this study or to the differences in ethnicity, inclusion and exclusion parameters, residence and existence of haplotype blocks with a special combination of other variants in risk loci. According to Yaghoobi et al., the *TMPRSS2* gene has various SNPs and their combinatory effects may Influence the disease outcome in a complex way, causing these conflicting results.[7] More investigation is needed to clarify this issue. Since the first evidence demonstrating the importance of *TMPRSS2* in SARS-CoV-2 entry,[2] several studies have been conducted in order to investigate the association between genetic polymorphisms in *TMPRSS2* and the risk of COVID-19, using genomic databases.[9,16–19] Among the various SNPs in the *TMPRSS2* gene, some have shown promise, as they are associated with changes in *TMPRSS2* expression levels, including rs2070788 and rs12329760.[19–21]

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In the current study, *TMPRSS2* mRNA folding in the nasopharyngeal tissue of severe COVID-19 patients was significantly less than that of control. While no significant difference was found in the *TMPRSS2* expression folding between the three genotypes, a higher expression was found in the CC genotype followed by CT and TT. Irham et al. reported that rs2070788 with GG genotype had the highest expression in the lung as compared to heterozygous AG and AA genotypes,[19] which was in agreement with previous results. The G allele frequency for rs2070788 appeared to be lower in the Asian populations as compared to the European and American populations. Overall, the frequencies of variant alleles (rs464397, rs469390, rs2070788 and rs383510) associated with high *TMPRSS2* expression in the lung seemed to be lower in the East Asian populations as compared to the European, African, and American populations. Thus, the European, African, and American populations may have more individuals with elevated *TMPRSS2* expression, which might lead to a higher susceptibility to COVID-19. Recognising the importance of the *TMPRSS2* gene in the SARS-CoV-2 infection cycle, the disparity in COVID-19 infection and severity could be attributed to the enhanced *TMPRSS2* gene expression. The functional basis of rs2070788 is still not understood, since rs2070788 is not located in any known regulatory regions.[22] Thus, variation in this gene may play an important part and may result in a difference in disease susceptibility in different communities around the world. Finally, the molecular technique should be applied in different areas related to medicine such as diagnosis of cancers or infectious diseases.[23–41]

Conclusion

This study showed a statistically significant association between the rs2070788 TT genotype and T allele as a risk factor for susceptibility to severe COVID-19 in the Iraqi population. In addition, the expression of the *TMPRSS2* gene revealed dysregulation (down-regulation) in severe patients. More sample sizes should be used in subsequent studies to confirm the findings of this study.

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Conflict of Interest: None

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