Research, Society and Development, v. 11, n. 15, e03111536658, 2022
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v11i15.36658

Relation of *TGF-β1* gene with the prognosis of patients with Covid-19

Relação do gene *TGF-β1* com o prognóstico de pacientes com Covid-19

Relación del gen *TGF-β1* con el pronóstico de pacientes con Covid-19

Received: 10/18/2022 | Revised: 10/29/2022 | Accepted: 11/01/2022 | Published: 11/08/2022

Christiane Ruffato Carminati
ORCID: https://orcid.org/0000-0003-2334-4570
Universidade Federal do Triângulo Mineiro, Brazil
E-mail: chriscarminati@gmail.com
Anna Cecília Dias Maciel Carneiro
ORCID: https://orcid.org/0000-0003-4865-1286
Universidade Federal do Triângulo Mineiro, Brazil
E-mail: annaceciliamaciel@gmail.com
Andrezza Cristina Cancian Hortolani da Cunha
ORCID: https://orcid.org/0000-0001-8800-4783
Universidade Federal do Triângulo Mineiro, Brazil
E-mail: andrezzacancian@hotmail.com
Loren Queli Pereira
ORCID: https://orcid.org/0000-0003-2891-443X
Universidade Federal do Triângulo Mineiro, Brazil
E-mail: lorenbiomedica@gmail.com
Fernanda Bernadelli De Vito
ORCID: https://orcid.org/0000-0002-7407-4972
Universidade Federal do Triângulo Mineiro, Brazil
E-mail: fernanda.vito@uftm.edu.br
Marcos Vinícius da Silva
ORCID: https://orcid.org/0000-0002-2966-7621
Universidade Federal do Triângulo Mineiro, Brazil
E-mail: marcosuftm@gmail.com
Virmondes Rodrigues Júnior
ORCID: https://orcid.org/0000-0001-8706-4223
Universidade Federal do Triângulo Mineiro, Brazil
E-mail: virmondes.rodrigues@uftm.edu.br
Mariângela Torreglosa Ruiz Cintra
ORCID: https://orcid.org/0000-0002-8223-805X
Universidade Federal do Triângulo Mineiro, Brazil
E-mail: mariangela.cintra@uftm.edu.br
Roseane Lopes da Silva Grecco
ORCID: https://orcid.org/0000-0002-9823-2074
Universidade Federal do Triângulo Mineiro, Brazil
E-mail: roseane.grecco@uftm.edu.br
Sarah Cristina Sato Vaz Tanaka
ORCID: https://orcid.org/0000-0003-4466-6093
Universidade Federal do Triângulo Mineiro, Brazil
E-mail: sarahtanaka20@hotmail.com
Helio Moraes de Sousa
ORCID: https://orcid.org/0000-0001-8049-348X
Universidade Federal do Triângulo Mineiro, Brazil
E-mail: helio.moraes@uftm.edu.br

Abstract

This study aimed to investigate the role of the TGF-β1 gene in SARS-CoV-2 infection. A total of 178 individuals diagnosed with Covid-19 participated and, they were divided in two groups related to the outcome (discharge or death). Genotyping of rs1800468 and rs1800469 polymorphisms of TGF-β1 gene was performed in 178 samples, using the allelic discrimination technique and, gene expression analysis was performed in 93 samples by Real Time PCR. There was no association between the genotypic frequencies of TGF-β1 gene polymorphisms analyzed with the prognosis of patients with Covid-19. There was no significant difference between gene expression and the clinical data evaluated. A statistically significant difference was observed in the expression of the TGF-β1 gene between the CT and TT genotypes of the rs1800469 polymorphism, with lower gene expression in the presence of the TT genotype. Regarding the rs1800468 polymorphism, no statistically significant difference was observed in the expression of the TGF-β1 gene in relation to the analyzed genotypes. The present study concluded that the rs1800468

and rs1800469 polymorphisms of the TGF-β1 gene are not associated with the prognosis of patients with Covid-19 and which the TT genotype of the rs1800469 polymorphism reduces the expression of TGF-β1.
Keywords: Covid-19; Cytokines; Genetic polymorphism; SARS-CoV-2; TGF-β1.

Resumo

Este estudo teve como objetivo investigar o papel do gene TGF-β1 na infecção por SARS-CoV-2. Participaram 178 indivíduos diagnosticados com Covid-19, que foram divididos em dois grupos relacionados ao desfecho (alta ou óbito). A genotipagem dos polimorfismos rs1800468 e rs1800469 do gene TGF-β1 foi realizada em 178 amostras, utilizando a técnica de discriminação alélica e, a análise da expressão gênica foi realizada em 93 amostras por Real Time PCR. Não houve associação entre as frequências genotípicas dos polimorfismos do gene TGF-β1 analisados com o prognóstico de pacientes com Covid-19. Não houve diferença significativa entre a expressão gênica e os dados clínicos avaliados. Foi observada diferença estatisticamente significativa na expressão do gene TGF-β1 entre os genótipos CT e TT do polimorfismo rs1800469, com menor expressão gênica na presença do genótipo TT. Em relação ao polimorfismo rs1800468, não foi observada diferença estatisticamente significativa na expressão do gene TGF-β1 em relação aos genótipos analisados. O presente estudo concluiu que os polimorfismos rs1800468 e rs1800469 do gene TGF-β1 não estão associados ao prognóstico de pacientes com Covid-19 e que o genótipo TT do polimorfismo rs1800469 reduz a expressão do TGF-β1.
Palavras-chave: Covid-19; Citocinas; Polimorfismo genético; SARS-CoV-2; TGF-β1.

Resumen

Este estudio tuvo como objetivo investigar el papel del gen TGF-β1 en la infección por SARS-CoV-2. Participaron un total de 178 personas diagnosticadas con Covid-19 y se dividieron en dos grupos según el desenlace (alta o muerte). Se realizó el genotipado de los polimorfismos rs1800468 y rs1800469 del gen TGF-β1 en 178 muestras, utilizando la técnica de discriminación alélica y, el análisis de expresión génica se realizó en 93 muestras por Real Time PCR. No hubo asociación entre las frecuencias genotípicas de los polimorfismos del gen TGF-β1 analizados con el pronóstico de los pacientes con Covid-19. No hubo diferencia significativa entre la expresión génica y los datos clínicos evaluados. Se observó una diferencia estadísticamente significativa en la expresión del gen TGF-β1 entre los genotipos CT y TT del polimorfismo rs1800469, con menor expresión génica en presencia del genotipo TT. En cuanto al polimorfismo rs1800468, no se observó diferencia estadísticamente significativa en la expresión del gen TGF-β1 en relación a los genotipos analizados. El presente estudio concluyó que los polimorfismos rs1800468 y rs1800469 del gen TGF-β1 no están asociados con el pronóstico de los pacientes con Covid-19 y que el genotipo TT del polimorfismo rs1800469 reduce la expresión de TGF-β1.
Palabras clave: Covid-19; Citocinas; Polimorfismo genético; SARS-CoV-2; TGF-β1.

1. Introduction

The Corona Virus Disease 19 (Covid-19) was initially related in Wuhan, China, in the end of December 2019, as a pneumonia of unknown cause. The causative agent was quickly identified as a β-coronavirus genera, was then called SARS-CoV-2 because of its closed relation with severe acute respiratory syndrome (SARS) (Rai et al., 2021). This disease, transmitted mainly by respiratory droplets, has a rapid spread, which made the World Health Organization (WHO) officially declare Covid-19 as a public health emergency of international concern in early 2020 (Karunasagar, 2020).

The clinical spectrum of this disease variates of asymptomatic to symptomatic cases and, the severe complications are more frequently in elderly patients and/or with associated comorbidities (diabetes mellitus, cardiovascular diseases, obesity and kidney diseases), suggesting which inefficient immune responses can play an important role in infection outcome (Ferreira-Gomes *et al*.,2021; Khadke *et al.*, 2020). The Covid-19 has a particular feature, mainly in severe cases, which is an exacerbated inflammatory response, where abnormal levels of different cytokines such as IL6, IL8, TNF-α e TGF-β1 have already been related (Costela-Ruiz et al., 2020; Di Maria et al., 2020).

The TGF-β1 is a multifunctional cytokine with regulatory functions including proliferation, differentiation, migration and apoptosis (Morikawa et al., 2016. It performed an important role in tissue repair after lesions in multiple organs, including lungs, where its acting in tissue repair is fundamental for the fibroproliferative response development (Shull, *et al.*, 1992; Broekelmann et al., 1991). The literature shows which TGF-β1 has a crucial role in respiratory complications caused by Covid-19, because it promotes the activation of bronchial mucosal cells and inducing the production of large amounts of thick

mucus and pulmonary. Also, the clinical manifestations of this disease are highly consistent with the increase of TGF-β1 activity, reinforcing its key role in the cytokine storm resulting of SARS-CoV-2 infection (Shen et al., 2021).

The TGF-β1 gene is localized in 19q13.2 chromosome and genetic variants in this gene have been associated with susceptibility to several diseases such as rheumatoid arthritis, hypertension (Li *et al.*, 1999), multiple sclerosis (HE,1998) and, osteoporosis (Yamada *et al.*, 2000). It is believed which the individual genetic profile plays an important role in determining the clinical response to the virus. However, the establishment of molecular mechanisms involving TGF-β1 are still not fully understood and require further studies (Casanova, *et al.*, 2020). Therefore, the aimed of this study is to evaluate the role of TGF-β1 gene in the prognostic of patients infected by SARS-CoV-2.

2. Methodology

Ethical considerations

This study was approved by the Research Ethics Committee of Clinical Hospital of the Federal University of Triângulo Mineiro (CAAE 31328220.8.0000.8667), and the participants signed the consent form.

Sample characterization

This is an observational, cohort study. A total of 178 patients diagnosed with Covid-19 were included, according to the guidelines established by the Ministry of Health (BRASIL, 2020) treated in hospital units in the city of Uberaba-MG, from May 2020 to November 2021. Data such as age, pre-existing diseases, period of hospitalization, outcome (death or discharge) and severity were obtained through a survey of medical records. To define severity, the following parameters were used: mild condition – patient in good general condition, saturation above 95%, without the need for mechanical ventilation; severe condition – patient requiring mechanical ventilation.

DNA Extraction and Genotyping

Samples of 10mL of peripheral blood were collected by venipuncture, in sterile collection tubes containing ethylenediaminetetraacetic acid (EDTA). The patients genomic DNA were extracted using Wizard® Genomic DNA Purification kit (PROMEGA® – EUA), according to manufacturer recommendations.

The genotyping of TGF-β1 gene and, the polymorphisms rs1800468 and rs1800469 were performed in 178 samples, by the allelic discrimination technique with TaqMan® SNP Genotyping Assays System (ABI, EUA) by Real-time PCR using the 7500 equipment (ABI, EUA). The probes and the primers were designed by Applied Biosystems (Assay ID: C__8708473_10; C__8708474_20). The PCR reactions were performed according to the manufacturer recommendations.

RNA Extraction and Gene expression

The RNA extraction was performed in 93 samples, using extraction kit SV Total RNA Isolation System (PROMEGA - EUA), according to manufacturer recommendations. The reverse transcription for cDNA obtaining were performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, EUA), according to manufacturer instructions.

Relative quantification of the TGF-β1 gene was performed by real time PCR, using the 7500 Real Time PCR System (Applied Biosystems™) equipment. The ACTB reference gene was used as an endogenous control. Assays containing specific primers and probes for each gene (ACTB and TGF-β1) were pre-designed by Applied Biosystems (Hs99999903_m1 and Hs00998133_m1, respectively) and performed according to the manufacturer recommendations. Relative quantification (RQ)

was performed by the $2^{-\Delta Ct}$ method (Schmittgen & Livak, 2008), where the threshold cycle (Ct) is the cycle in which the fluorescence level reaches sufficient quantity for sample detection.

Statistical Analysis

Continuous variables were described as mean ± standard deviation and categorical variables were expressed as percentage. Statistical comparisons between two groups were performed using the Mann-Whitney test and for comparisons between more than two groups the Kruskal Wallis test was used. For qualitative variables, statistical comparisons were performed using the chi-square test. The statistical significance was defined as $p<0.05$.

3. Results

Sample characterization

In this study, 178 patients diagnosed with Covid-19 participated. It was 108 men (60,7%) and 70 women (39,3%) participated. The mean age was 59 years (±17,5) and was a statistical significative difference relative to age and the outcome (death) of patients, p=0.001. The ethnicity of patients was reported in 136 cases, as follows: white (59%), oriental (3,6%), black (3,6%) and, mixed race (33,8%). Regarding the outcome of the patients, it was found that 90 died (50.6%) and 88 patients were discharged (49.4%). Regarding comorbidities, 131 (73.6%) had one or more comorbidities: 82 with systemic arterial hypertension, 42 with diabetes mellitus, 75 with other comorbidities (obesity, hypothyroidism, asthma), and 47 had no comorbidities. There was a significant difference between the outcome (death) of patients with comorbidities,p=0.005.

Analysis of TGF-β1 gene polymorphisms (rs1800468 and rs1800469)

The polymorphisms rs1800469 and rs180046 in the TGF-β1 were analyzed in 178 samples. The genotypical and allelic frequencies to the polymorphisms compared to the outcome of patients are presented in the Table 1. No statistically significant difference was observed between genotypic frequencies and patient outcomes for the investigated polymorphisms (p=0.248 and p=0.921). Both polymorphisms are in Hardy Weinberg Equilibrium: rs1800468 (High Group: $\chi2=0.56$, p=0.45; Death Group: $\chi2=0.31$, p=0.58); rs1800469 (High Group: $\chi2=0.0014$, p=0.97; Death Group: $\chi2=0.90$, p=0.34).

Table 1 - Distribution of genotypic and allelic frequencies of rs1800469 C>T and rs1800468 G>A polymorphisms in the TGF-β1 gene in relation to outcome.

Polymorfism	Discharge n(%)	Death n(%)	p
rs1800468			
GG	75 (85,2)	80 (88,9)	
GA	13 (14,8)	10 (11,1)	0,248
AA	0	0	
rs1800469			
CC	11(12,5)	12 (13,3)	
CT	40 (45,5)	36 (40,0)	0,921
TT	37 (42,0)	42 (46,7)	
Allelic frequencies			
rs1800468			
G	163(49,0)	170 (51,0)	
A	13 (56,6)	10 (43,4)	
rs1800469			
C	62 (50,9)	60 (49,1)	0,79
T	114 (48,7)	120 (51,3)	

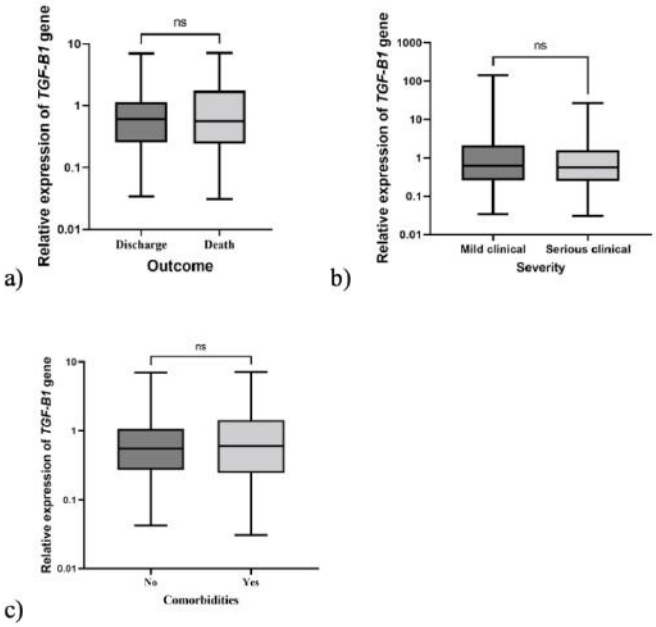
Chi-squared test. Source: Authors.

TGF-β1 gene expression

Comparison between TGF-β1 gene expression and clinical data

The relative quantification of the TGF-β1 gene was performed in 93 samples. TGF-β1 gene expression was compared for outcome (discharge and death), severity (mild and severe) and, presence or absence of comorbidities. There were no significant differences between gene expression and the evaluated data (p=0.65, p=0.65 and p=0.53) (Figure 1).

Figure1 – Comparision between TGF-β1 gene expression and: (**a)** patient's outcome, **(b)** severity of cases, **(c)** presence or absence of comorbidities. Mann-Whitney test.

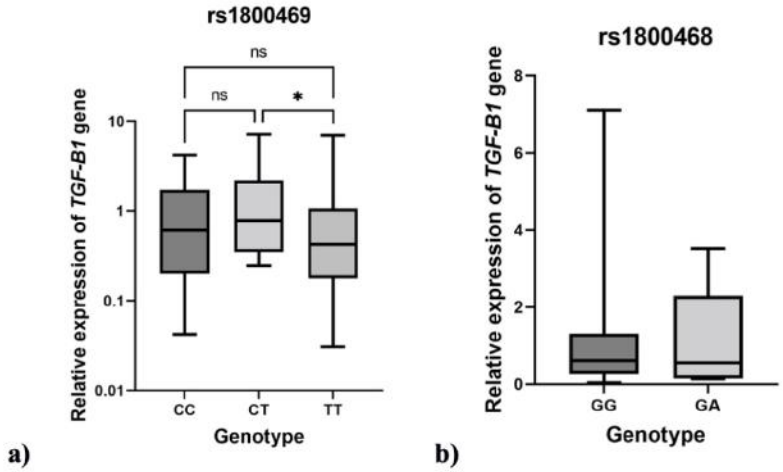


Source: Authors.

Comparison between TGF-β1 gene expression and genotypes of polymorphisms rs1800468 and rs1800469

The expression of the TGF-β1 gene was compared with the genotypes of the rs1800469 and rs1800468 polymorphisms. A statistically significant difference was observed in the expression of the TGF-β1 gene between the CT and TT genotypes of the rs1800469 polymorphism, with lower gene expression in the presence of the TT genotype (p=0.03). In contrast, in the rs1800468 polymorphism, no statistically significant difference was observed in the expression of the TGF-β1 gene in relation to the GG and GA genotypes (p= 0.91) (Figure 2).

Figure 2 – Comparison between TGF-β1 gene expression and genotypes of the rs1800469 C>T polymorphism. Kruskal Wallis test.



Source: Authors.

4. Discussion

Comorbidities are conditions which actively affect manifestations and clinical outcomes of patients infected with SARS-CoV-2. Tian and collaborators (2020) evaluated data from 13.077 individuals with Covid-19 at nine hospitals of Wuhan, China, between January and March 2020. They observed which diabetes, hypertension and cardiovascular disease were highly prevalent among patients, predisposing them to poor clinical outcomes (Tian *et al.*, 2020).

Similarly, a systematic review realized with data of 18.012 patients with Covid-19 concluded which diabetes, hypertension and cardiovascular diseases are important risk factors for severity and mortality in people infected (de Almeida-Pititto *et al.*, 2020). These data are in line with those observed in the present study, where it was found that hypertension and diabetes were the predominant comorbidities, demonstrating the influence of certain risk factors on the outcome of the infection.

Considering which TGF-β1 plays an important task in innate and acquired immunity (Wahl et al., 2006) and, rs1800468 and rs1800469 polymorphisms regulate protein expression levels (Luedecking *et al.*, 2000), increasing and decreasing, respectively, the production of TGF-β1 (Loeys *et al.*, 2006; Grainger *et al.*, 1999), we expected to find correlation between the presence of these polymorphisms and disease severity, which was not observed.

However, although the present study did not find an association between the evaluated polymorphisms and Covid-19, the influence of genetic polymorphisms on the susceptibility to SARS-CoV-2 infection has been demonstrated. Gómez and collaborators found an association between the rs2285666 polymorphism of ACE2 gene and the risk of developing severe Covid-19, in a study realized in Spain with 204 patients (137 non-severe cases and 67 severe ICU cases) and 536 controls paired by age. That study suggested that *ACE-ID* polymorphism was associated to the risk of severe Covid-19 development,

depending on hypertension status. The ACE rs2285666 variant was associated with hypertension in the elderly population, with no significant difference between patients with Covid-19 mild and severe (Ferreira-Gomes *et al.*, 2020).

Another example is a study conducted by KIM and collaborators in South Korea to find genetic biomarkers for Covid-19 severity. It was evaluated a populational-level correlation between Covid-19 fatality rate and genetic polymorphisms of several potential genes related to Covid-19, including IFITM3, ACE2, TMPRSS2, IL6, LZTFL1 and, the ABO genes. These correlations were investigated with the numbers of Covid-19 cases and deaths from the WHO Covid-19 panel and calculated the fatality rate for each ethnic group. The allelic frequencies of polymorphisms evaluated were obtained through the 1000 Genomes Project. Three SNPs of the IFITM3 gene, rs12252, rs34481144, and rs6598045, were strongly correlated with mortality. This gene is classified as a member of small interferon-stimulated genes for (ISGs) and plays a primordial role in defense against enveloped viruses, including influenza A virus (Kim & Jeong, 2020).

The influence of TGF-β1gene polymorphisms, rs1800468 and rs1800469, and Covid-19 has not available data yet however it has already been analyzed in other diseases. Juarez and collaborators in Spain (2021) evaluated these same polymorphisms in plasma samples from 108 patients with gastric adenocarcinoma and observed an association between the G/A genotype of the rs1800468 polymorphism and the worse disease prognostic. It was also noticed which this polymorphism promotes lower production of TFG-β1, increasing chances of metastasis development and, concluded that these SNPs can help to identify patients at risk of developing advanced disseminated disease and shorter life expectancy, requiring surgical and therapeutic more aggressive approach from the beginning (Juarez *et al.*, 2021).

A review study, by meta-analysis, investigated a potential correlation between the TGF-β1 gene rs1800469 polymorphism and lung cancer. This study concluded which this polymorphism is not implicated in lung cancer susceptibility in the general population, however, the analysis indicated that it decreases the risk of lung cancer in patients with NSCLC (non-small cell lung cancer). Individuals with the TT genotype have a slightly lower incidence of lung cancer compared to individuals with the other genotypes, suggesting a possible protective role of the TT variant for lung cancer susceptibility in patients with NSCLC. (Chen *et al.*, 2019).

In the present study, no difference was found between the expression levels of the TGF-β1 gene and the outcome, severity or presence of comorbidities in patients with Covid-19. However, a study carried out by Villalba et al. (2020), in Cuba, with 45 patients positive for SARS-CoV-2 (divided into two groups: asymptomatic and symptomatic) and 20 controls, evaluated the early inflammatory response in the upper airways through the analysis of the expression of IFN-γ, TGF-β1 and RANTES genes. It was demonstrated which the expression of TGF-B1 was significantly lower in patients positive for SARS-CoV-2 than in the control group. According to symptoms, the concentration of TGF-β1 mRNA did not differ significantly between the groups, although the expression was lower in asymptomatic people compared to symptomatic cases (Montalvo Villalba *et al.*, 2020).

A previous study by Ghazavi and colleagues in Iran, with 63 adult patients with Covid-19 and 33 healthy individuals matched for gender and age, showed that TGF-β1 increased significantly in patients with the severe form of the disease, possibly due to the infiltration of immune cells. in the lungs, which can release TGF-β1 into the blood of patients (Ghazavi *et al.*, 2020). Zheng et al., in a study carried out in China with 285 patients with sepsis (119 patients with severe sepsis and 166 patients with mild sepsis) and 285 healthy individuals (control group) found plasma levels of TGF-β1 higher in patients with severe sepsis, indicating that the higher concentration of this cytokine contributes to the worsening of the condition (Zheng, Fu & Zhao, 2021).

Regarding the genotypes of the rs1800468 and rs1800469 polymorphisms and the expression of the TGF-β1 gene, in the present study it was observed that individuals with the TT genotype of the rs1800469 polymorphism expressed

significantly lower amounts of TGF-β1 compared to the other genotypes. Conversely, JUAREZ et al. observed lower amounts of TGF-β1 in individuals with the CC genotype of the rs1800469 polymorphism. As for the rs1800468 polymorphism, no homozygous mutant was identified and no statistically significant difference was observed in gene expression between the GG and GA genotypes.

A study carried out in China in patients with sepsis associated the rs1800469 polymorphism of the TGF-β1 gene to lower plasma levels of this cytokine and a reduced risk of susceptibility to sepsis. However, the same study observed that the rs1800468 polymorphism was associated with higher levels of TGF-β1 and greater susceptibility to sepsis (ZHENG, et al. 2021), a result that is different from the result found in our study.

The fact that the present study had not found an association between the rs1800469 and rs1800468 polymorphisms of the TGF-β1 gene and the outcome, nor with the gene expression with the outcome, severity, or presence of comorbidities in individuals with Covid-19, can be attributed to the complex network of interaction in the activation of the immune response to infection. Thus, the broad phenotype observed in patients infected with SARS-CoV-2 is possibly the result of inter-individual, genetic, and acquired differences, which influence the clinical course of the disease, where different patients with Covid-19 and even the same patient at different stages of the disease can present diverse pathogenesis and clinical manifestations.

As limitations of the study, the sample size and the scarcity of data in the literature on the contribution of the TGF-β1 gene and its polymorphisms to the prognosis of patients with Covid-19 made it difficult to interpret the data found. However, we highlighted the novelty of this work in evaluating the rs1800468 and rs1800469 polymorphisms of TGF-β1 gene and its expression in individuals with Covid-19 in a Brazilian population. Thus, more studies comparing the expression of TGF-β1 at different times of infection, in addition to studies which evaluate the influence of polymorphisms in this gene in different ethnic groups are necessary to better characterize the role of molecular alterations involving this gene in the pathogenesis of the disease.

5. Conclusion

This study concluded that there is no association between rs1800468 and rs1800469 *TGFβ1* polymorphisms with prognostic of patients with Covid-19. Besides, the presence of comorbidities, severity and outcome did not influence the gene expression of *TGFβ1* in the samples evaluated. Thus, we concluded that individuals with TT genotype of rs1800469 polymorphism has lower levels of *TGFβ1*. Despite our results, further studies are needed to conclude the importance of these polymorphisms with the prognosis of patients with Covid-19.

Financial Support

CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior / Programa estratégico Epidemias – Edital emergencial 09/2020 (668/2020).

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