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EXPERIMENTAL PAPERS

RNA N6-Methyladenosine Pathway Writer Genes Expression Levels and Clinical Severity of Infection in Covid-19 Patients

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Abstract—Epigenetic modifications are known to be effective in the severity and mortality rate of SARS-CoV-2 infection. N6-methyladenosin (m6A) is a posttranscriptional modification that is carried out by m6A methyltransferases (METTL3, METTL14, and WTAP). This modification is effective in the formation of a natural immune response in the relationship between the viral genome and the host cell. In this study, the relationship between clinical severity and METTL3, METTL14, WTAP expression levels in Covid-19 patients was studied for the first time. Also, patients' D-dimer, ferritin, and C-reactive protein values were compared with these gene expression levels. Total RNA was extracted from blood samples of 100 volunteers and gene expressions were measured using a quantitative real-time polymerase chain reaction. It was determined that METTL3 ($p < 0.001$) and METTL14 ($p = 0.005$) genes were statistically significant between case and control. In addition, METTL14 ($p = 0.007$) and WTAP ($p = 0.015$) gene expressions were significantly increased in patients with severe disease. METTL14 was statistically significant between the male patients and the control (fold change = 63.87, $p = 0.015$). Overexpression of the METTL14 gene may have resulted in higher clinical severity in males. Our results demonstrate that host N6-methyladenosine (m6A) methyltransferases may be effective in the development of SARS-CoV-2 infection and prognosis of the disease.

Keywords: COVID-19, N-methyladenosine, Methyltransferases, Clinical severity

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1. BACKGROUND

SARS-CoV-2 has become a global health problem in a short time due to its rapid spread and high mortality rate. Viral infection pathogenesis can be affected by m6A modification of viral or host transcripts [1]. It is known that the interaction between host and viral genomic RNA is important in the severity and survival rate of viral infections [1, 2]. Few studies have shown that SARS-CoV-2 genomic RNA is dynamically modified with N6-methyladenosin (m6A) [2, 3]. Functional experiments have shown that host m6A methylation negatively regulates SARS-CoV-2 infection and may also be associated with disease severity and survival [2–4].

The full genome of Wuhan-Hu-1 coronavirus (WHCV), a SARS-CoV-2 strain isolated from a COVID-19 pneumonia patient, is 29.9 kb and has a poly (A) tail at its 3' end and a hat structure at its 5' end. This feature enables it to act as an mRNA for the translation of replicase proteins [5]. Mainly two-thirds of viral RNA encodes pp1a, pp1ab proteins, and 16 nonstructural proteins (NSPs) first of all ORF (ORF1a/b), other ORFs encode structural and accessory proteins. The remaining virus genome encodes for four essential structural proteins, a small envelope protein (E), spike glycoprotein (S), nucleocapsid protein (N), matrix protein (M), and several auxiliary proteins [6]. Based on evolutionary analyzes of genome sequencing results, SARS-CoV-2 is thought to have been transmitted from bats to humans through unknown intermediate hosts [7].

The SARS-CoV-2 genome contained m6A modification at a rate of 0.096% and approximately eight regions carried m6A modification. The 3' end of the

SARS-CoV-2 RNA was found to be rich in N6-methylation of adenosine (m6A) modification. SARS-CoV-2 can escape the host immune response via the m6A modification it carries [8, 9]. The transcriptome analysis of the m6A methyloma of SARS-CoV-2 has shown that m6A methyltransferases and demethylases play a role in the regulation of the viral life cycle and m6A has a viral suppressive role. The host m6A methyloma, including the m6A location and methylation motifs, changes after SARS-CoV-2 infection [4]. METTL3 has proven to regulate viral m6A RNA modification and to play a role in the formation of the host innate immune response in SARS-CoV-2 infection. The m6A has been identified as a dynamic epitranscriptomic sign mediating virus–host interactions [9]. The host m6A modification complex regulates SARS-CoV-2 replication by interacting with viral proteins [3]. When the host cell encounters the virus, the host's METTL3 adds the m6A modification to the SARS-CoV-2 viral RNA. METTL3 and METTL14 genes expression levels increases in the host cell cytoplasm [8]. According to an investigation, viral replication rate increase in METTL3 and METTL14 knockdown host cells. It was concluded that the host m6A RNA modification negatively regulates the SARS-CoV-2 life cycle [4]. The METTL3, METTL14 and WTAP genes are the most important N6-methyladenosine writer genes in viral diseases according to the current literature. While several viruses alter m6A modification in cellular mRNAs the role of m6A in cellular mRNA during viral infection is still not well understood. In this study, we aimed to investigate the METTL3, METTL14 and WTAP genes expression levels in Covid-19 patients, and healthy controls. In addition, we analyzed the relationship between this genes expression levels in clinical severity and genders.

2. METHOD

Blood samples were taken from 60 patients diagnosed with Covid-19 in the Infectious Diseases and Clinic Microbiology Department of Sivas Cumhuriyet University Hospital. The severity of patients was classified according to the COViD-19 adult patient treatment guide of the Republic of Turkey Ministry, Health General Directorate of Public Health. The control group was recruited before the Covid-19 pandemic, from 40 healthy individuals who were not diagnosed with any infection. The informed consent form was obtained from the individuals. Samples taken into Paxgene blood RNA tubes were kept at −20°C until total RNA isolation. Total RNA was isolated using the PAXgene blood RNA kit. RNA concentration was measured using the QuantiFluor RNA System. Complementary DNA (cDNA) synthesis was performed using the cDNA synthesis kit (A.B.T, Cat. no. C03-01-20). Real-Time qPCR reaction was performed with A.B.T. SYBR Green Master mix (A.B.T. Cat. no. Q03-02-05) using target primers of METTL3, METTL14, and WTAP genes. The GAPDH gene was used as an endogenous control gene. When quantifying mRNA expressions, the GAPDH transcript was used as a reference and normalized relative to the control group. The "ΔΔCt method" (also known as 2-ΔΔCt method) was used to calculate the relative quantification. The Geneglobe Qiagen Analysis Tool (10) was used to calculate fold change and p-values of target genes. Ct values over 35 cycles were considered undetectable.

3. RESULTS

In the study, the population was composed of 100 individuals (60 cases and 40 control). Patients' mean age was 62.27, 53.33% of them were female, and 46.66% them male. Healthy controls mean age was 35.03, female 59.37%, male 40.62%. It is not statisticaly significant age and gender between cases and controls. Patients were divided into three groups according to clinical severity severe, mild and moderate. When comparing the mean age of patients' according to clinical severity, there was a difference between severe (Mean age = 74.6 ± 10.37) and moderate/mild (Mean age = 56.33 ± 19.07) ($p = 0.001$).

METTL3, METTL14, and WTAP RNA expression levels were compared between the case and control groups. METTL3 gene expression was statistically significant ($p < 0.001$, fold change 0.26). METTL14 fold change was determined to be 25.59, which was also statistically significant ($p = 0.005$). The WTAP gene expression was determined to increase 4.01-fold, but the alteration was not significant ($p = 0.488$). Fold changes and p values are given in Table 1 and Fig. 1. GAPDH Ct means ± SD value was 31.71 ± 1.12 in the case group also, 26.58 ± 2.32 in the control group. METTL3, METTL14, and WTAP Ct mean ± SD values were 33.20 ± 1.36, 33.89 ± 2.59, and 31.57± 2.39 in cases, were 26.02 ± 2.38, 33.81 ± 4.37, 28.44 ± 2.77 in controls.

When compared to severe Covid-19 patients with moderate/mild METTL3 gene fold change was 1.46 ($p = 0.158$), METTL14 fold change was 3.58 ($p = 0.007$), and WTAP fold change was 3.86 ($p = 0.015$). The expression levels of the METTL14 and WTAP genes were significantly higher in severe than in moderate/mild disease. Also, severe Covid-19 patients were compared to mild Covid-19 patients, METTL3, METTL14 and WTAP fold changes were 1.16 ($p = 0.447$), 3.09 ($p = 0.062$), and 4.46 ($p = 0.088$) in respectively (Table 1, Fig. 1). Increased gene expression was observed in Covid-19 patients with high clinical severity compared to mild patients, but the increases were not statistically significant.

Gene expression levels were compared between the genders. There was no significant difference between male and female gene expression levels. METTL3, METTL14 and WTAP genes fold changes were 1.07

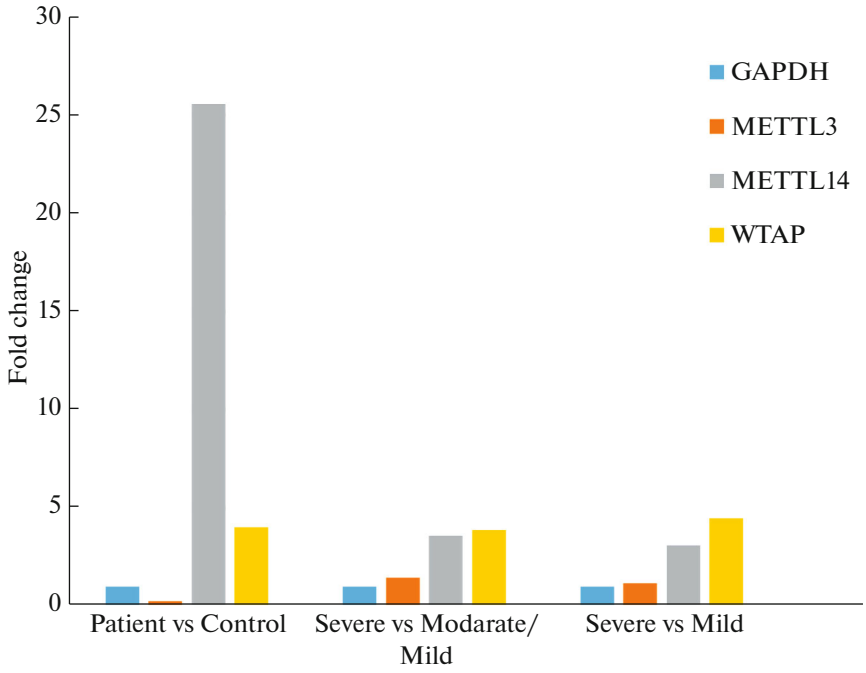

Fig. 1. Graphical representation of fold changes of METTL3, METTL14, and WTAP genes compared to healthy controls in COVID-19 patients. Multifold change graphs according to disease severity.

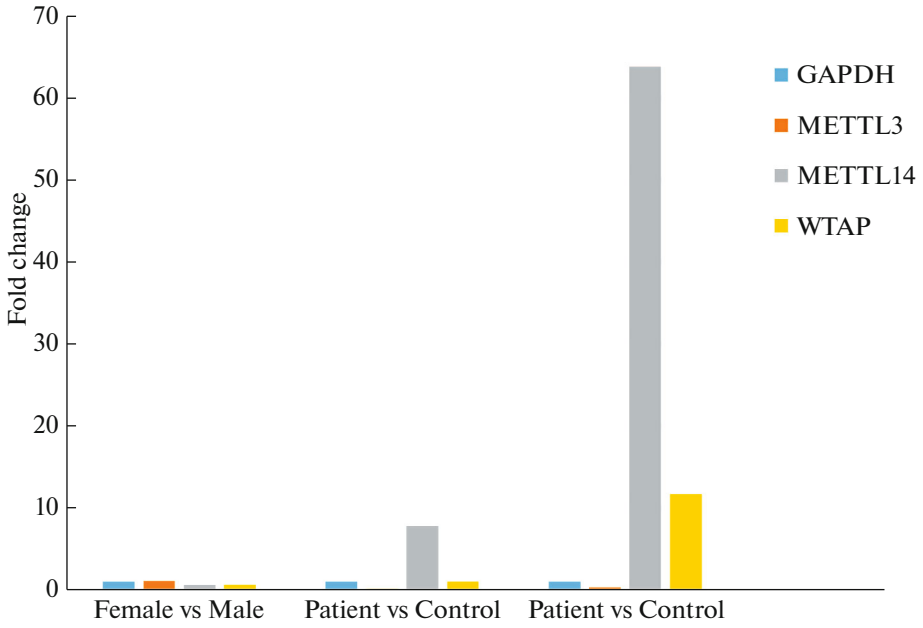

Fig. 2. Graphical representation of multifold change of METTL3, METTL14, and WTAP genes in females compared to males, female cases- female controls, and male cases–male controls.

($p = 0.832$), 0.66 ($p = 0.479$), and 0.63 ($p = 0.823$) in respectively. METTL3 gene expression levels were increased in female patients when compared with female controls (0.16-fold, $p < 0.001$). However, METTL14 (7.78-fold, $p = 0.165$) and WTAP (0.99-fold, $p = 0.983$) were not altered. When male patients were compared with male controls, the METTL14 gene was upregulated in male patients (63.87-fold, $p = 0.015$). METTL3 was also increased significantly (0.35-fold, $p < 0.001$). Also, WTAP was increased (11.69-fold, $p = 0.194$), but this alteration is not statistically significant (Table 2, Fig. 2).

Table 1. Comparison of writer genes in the m6A pathway in COVID-19 patients-healthy control and severity categories

	Patient vs Control		Severity (Severe vs Modarate/Mild)		Severity (Severe vs Mild)	
	fold change	*p*-value	fold change	*p*-value	fold change	*p*-value
METTL3	0.26	<0.001*	1.46	0.158	1.16	0.447
METTL14	25.59	0.005*	3.58	0.007*	3.09	0.062
WTAP	4.01	0.488	3.86	0.015*	4.46	0.088

*p values with significant fold change. METTL3 (p = <0.001) gene expression altered in patients. METTL14 (p = 0.005) gene expression increased excessively in patients. METTL14 (p = 0.007) and WTAP p = 0.015) genes expression levels increased significantly in severe disease compared to moderate/mild disease

Table 2. Fold changes in writer genes in the m6A pathway in females compared to males diagnosed with COVID-19. Comparison of fold changes in healthy females compared to diagnosed females. Comparison of fold changes in healthy males compared to diagnosed males

	Gender (Female vs Male)		Female (Patient vs Control)		Male (Patient vs Control)	
	fold change	*p*-value	fold change	*p*-value	fold change	*p*-value
METTL3	1.07	0.832	0.16	<0.001*	0.35	<0.001*
METTL14	0.66	0.479	7.78	0.165	63.87	0.015*
WTAP	0.63	0.823	0.99	0.983	11.69	0.194

*p values with significant fold change. METTL3 (p < 0.001) gene RNA levels altered in both genders when compared with their controls. METTL14 gene was overexpressed in male patients compared to male controls (63.87-fold, p = 0.015)

The patients' D-dimer, ferritin, and C-reactive protein values were compared with METTL3, METTL14, and WTAP gene expression levels. D-Dimers of all patients were above the normal range (>500 ng/mL). Therefore, any statistical comparisons could not be made for this parameter. When ferritin and C-reactive protein levels were compared with target gene expression levels, it was determined that METTL14 gene expression levels were increased in patients with high ferritin levels (p = 0.035) (Fig. 3). Also there was no statistical significance between C-reactive protein levels and target genes expression levels (Fig. 4). Statistical analyzes of C-reactive protein, ferritin, and target genes expression levels were given in Table 3.

DISCUSSION

M6A modifications and m6A methyltransferases play an important role in the host cell response and RNA virus life cycle [11, 12]. Some studies starting

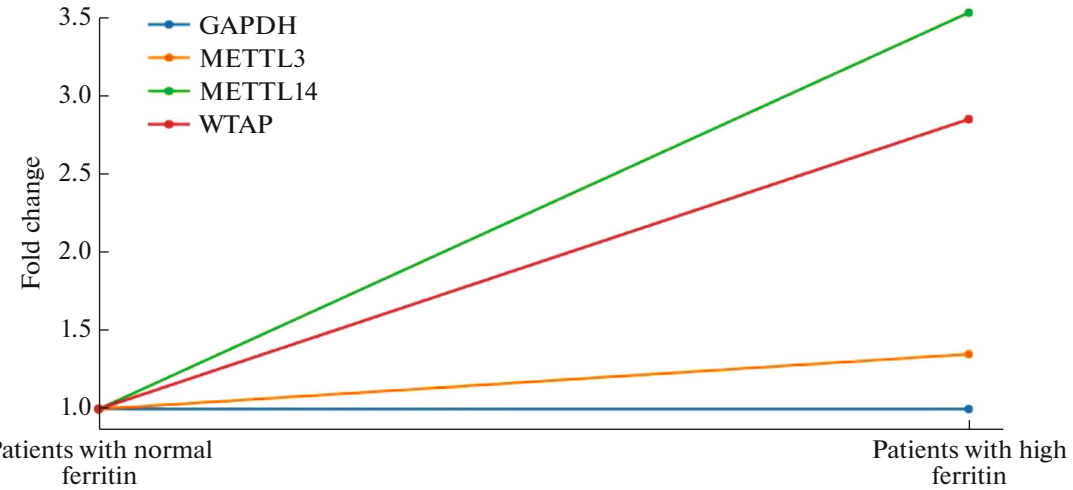

Fig. 3. Patients were classified with ferritin levels and compared with METTL3, METTL14, and WTAP expression levels. It was determined that METTL14 level was increased in patients with high ferritin levels (p = 0.035).

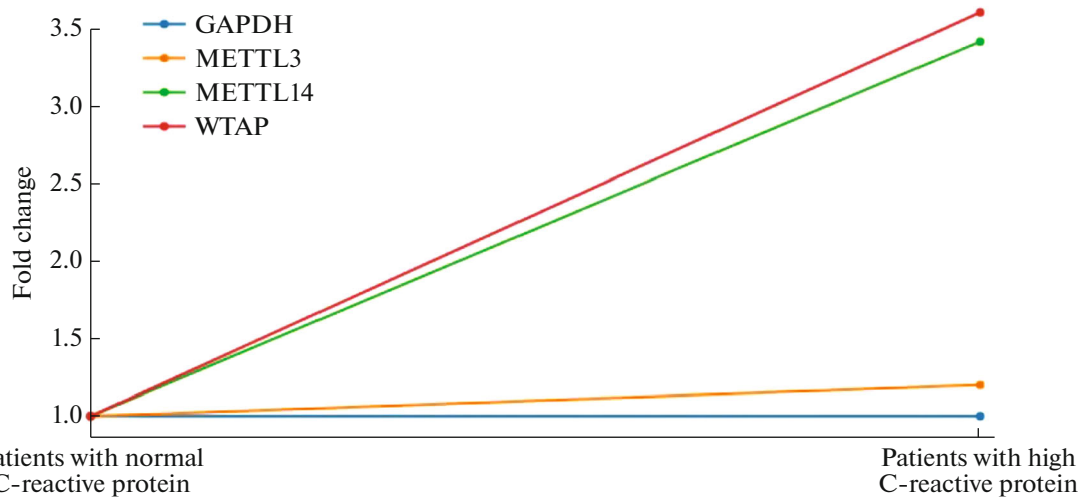

Fig. 4. Patients were classified according to high and normal C-reactive protein levels and compared with METTL3, METTL14, and WTAP expression levels. There were no statistical differences between the groups ($p > 0.05$).

with the COVID-19 pandemic, have shown that the m6A plays an important role in the progression of SARS-CoV-2 infection. Functional experiments have shown that m6A is a dynamic epitranscriptome that regulates SARS-CoV-2 infection which mediates virus-host interactions [3, 4]. The SARS-CoV-2 infection has been shown to cause dynamic changes in host m6A methylation. In the host cell infected with SARS-CoV-2, m6A modification is increased to modify the virus RNA (4). In the present study, we investigated METTL3, METTL14, and WTAP gene expressions in the host cells in SARS-CoV-2 infection. We also evaluated the expression levels of these methyltransferases in the clinical severity of the infection and between genders.

It is known that the METTL3 gene modifies the viral genome, preventing its replication and suppressing the innate immune response [9]. If METTL3 expression is insufficient in the host cell, viral genome m6A modification cannot be performed, viral genome replication increases, inflammatory genes expression increases, and a natural immune response occur. Chen et al., METTL3-mediated m6A modification in promoting antiviral immunity has been shown to be activated by innate signals and promote protein translation and mRNA stability. Also reported that innate immune signals regulate the m6A modification mechanism in response to viral infections. The type I IFN regulatory kinase TBK1 activates the m6A author core component METTL3 and has been identified to support innate immune responses in an m6A-dependent and -independent manner [11]. Zhang et al., showed that SARS-CoV-2 infection increased METTL3 expression in host cells. Modification of METTL3 expression in host cells with short hairpin RNA or silencing or increasing expression by plasmid transfusion also changed the virus replication [8]. In this study, we determined that the expression of the was 0.26 which ($p < 0.001$) gene was altered in Covid-19 patients compared to controls. Similar to previous studies, the METTL3 level was found to be statistically significant in Covid-19 patients in our study.

In the m6A methyltransferase complex, METTL3 plays a role as a catalytic subunit and METTL14 subunit. The METTL14 is known to regulate the immune response by activating germinal center B cells and stimulating the NF-κB/IL-6 signaling pathway in response to viral infection in the host cell [12]. Lang

Table 3. Covid-19 patients were divided into two groups according to high and normal levels of C-reactive protein and Ferritin

	Nonnal C-reactive protein/ High C-reactive protein		Nonnal ferritin/High ferritin	
	fold change	*p*-value	fold change	*p*-value
METTL3	1.20	0.506	1.35	0.495
METTL14	3.42	0.158	3.53	0.035*
WTAP	3.61	0.369	2.85	0.201

**p* values with significant fold change. There was a significant correlation between high ferritin levels and high METTL14 expression in patients

et al., Epstein–Barr virus identified the m6A modification of its lytic and latent transcripts, revealing the role of m6A in gene regulation of this virus. They determined that METTL14 expression levels were induced and knock-down of METTL14 led to decreased expression of latent EBV transcripts [13]. In herpes virus type 1 infection, it has been reported that the expression of METTL3 and METTL14 at the early infection stage increases to facilitate viral replication immediately after infection, and their expression decreases thereafter [14]. Zhu et al investigated the antiviral function of METTL14 and reported that manipulation of METTL14 could be a potential strategy to interfere with single-stranded RNA virus infections [15]. Also, we found that METTL14 ($p = 0.005$) gene expressions altered significantly in patients. We conclude that the expression of both METTL3 and METTL14 genes in the host genome can rapidly change in response to viral infection to control the viral genome.

WTAP activates the m6A methyltransferase complex by interacting with METTL3 and METTL14. RNA binding capacity of METTL3 decreases when WTAP is inhibited. Also, it is known to have a critical role as a regulator subunit in the m6A methyltransferase complex [16]. Matthew et al., reported that WTAP is required for both METTL3 interactions with HCV RNA and m6A modification throughout the viral RNA genome. They determined that after HCV infection, the localization of WTAP in the cytoplasm increased [17]. Xiao et al., reported that Epstein–Barr virus regulates WTAP by influencing the NF-κB signaling pathway, further WTAP influencing cell proliferation and migration in gastric carcinoma cells. Also found that EBV-encoded small RNA1 could downregulate WTAP expression by activating the NF-κB signaling pathway [18]. Our study determined that the alteration in the expression of the WTAP gene in patients was not significant. WTAP gene expression may not have increased sufficiently in patients due to the failure to establish the METTL3–METTL14 complex.

Host M6A methyltransferase has been shown to be involved in the progression of Covid-19 disease. Also, expression levels of these methyltransferases in the host cells effective in the clinical severity of the disease [2, 9]. In a microarray study conducted on Covid-19 patients with severe and mild disease, there were different m6A transcript profiles. In this study, Meng et al., reported that the duration of Covid-19 may be prolonged in patients with low methyl reserves, and the activity of the host M6A methyltransferase complex may be low in these patients [19]. According to our findings, there is no significant difference in METTL3 gene expression between different clinical severity. The METTL3 expression may be suppressed at a similar rate by viral genome modifications in all individuals where viral infection symptoms occur. The METTL14 expression may be overexpressed in all patients in response to viral infection and it may be increased more in those with severe disease than in others. The increase in the expression of the WTAP gene was found to be significant in patients with severe disease compared to others.

With the progression of the Covid-19 pandemic, Covid-19 progression and death rates were determined to differ between genders. It was observed that while females in business life were more frequently infected with Covid-19, the death rate was twice as high in male patients as in females [20]. In another study, it was determined that the risk of developing the disease was similar in males and females, but the clinical severity was higher in men and the mortality rate was 2.4 times higher [21]. Differences between genders due to X inactivation and the regulatory effects of sex hormones were thought to cause different clinical gender-specific modeling severities between genders. In addition, gender-specific behaviors, genetic and hormonal factors, and gender differences in biological pathways related to SARS-CoV-2 infection may be effective in the prognosis of the disease in males [21–23].

In our study, we determined that m6A methyltransferase expression levels were similar between genders. We found that the fold change in METTL3 expression was significant in both genders when compared to their controls, but the expression of the METTL14 gene increased 63.87 times in males. The role of METTL14 in the formation of the natural immune response has been demonstrated in recent studies [12, 24]. Overexpression of METTL14 may contribute to the severity and higher mortality in males. The METTL14 inhibition in men with severe disease can reduce mortality rates and can be shown to be a therapeutic target in men.

Ferritin, C-reactive protein, and D-dimer levels are also known to be important for the severity of Covid-19 patients [25, 26\. Serum high ferritin levels may trigger a cytokine storm in Covid-19 patients by exerting direct immunosuppressive and pro-inflammatory effects. D-Dimer is used to identify clots in the blood. C-Reactive protein increases in the body when there's inflammation. We compared the expression levels of target genes in patients with high and normal ferritin, C-reactive protein, and D-dimer levels. D-Dimer levels were high in all patients. Also, we determined no differences between C-reactive protein levels and target gene expression levels. We determined that high ferritin levels were associated with high METTL14 levels in patients ($p = 0.035$). Several studies have found an association between serum ferritin levels and the severity of COVID-19 disease [27, 28]. N6-methyladenosine modification has been reported to regulate ferroptosis through the autophagy signaling pathway in different cell types. It was reported that ferritin levels associated with the m6A-dependent mechanism [29]. Therefore, serum Ferritin levels and METTL14 gene expression may be correlated in patients.

CONCLUSION

In this study, we found that the expression of METTL3 and METTL14 was statistically significantly different in covid 19 patients compared to the control group. METTL14 and WTAP expression was significantly increased in patients with severe disease compared to those with moderate/mild disease. Also, the METTL14 gene was overexpressed in male patients compared to male controls. The N6-methyladenosine modification is important in the organization of infection-related pathophysiological processes. Host N6-methyladenosine methyltransferases METTL3 and METTL14 may be effective in the development of SARS-CoV-2 infection and prognosis of the disease. The METTL14 may be a therapeutic target for preventing poor prognosis in males. Increasing the host METTL3 reserve may be effective in preventing the disease in both genders. There may be a correlation between ferritin level and METTL14 expression. Further functional studies are needed to reveal the effect of these genes on the pathogenesis of Covid-19.

FUNDING

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COMPLIANCE WITH ETHICAL STANDARDS

Statement of Compliance with Standards of Research Involving Humans as Subjects

Ethics committee approval was received from Sivas Cumhuriyet University Clinical Research Ethics Committee (ethics committee decision no: 2020-10/01). The study was conducted in accordance with the Principles of the Declaration of Helsinki.

Informed consent was obtained from all individual participants included in the study.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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