Full Length Research Article
Advancements in Life Sciences – International Quarterly Journal of Biological Sciences

ARTICLE INFO

Date Received: 18/12/2024;
Date Revised: 02/02/2025;
Date Published Online: 28/12/2025;

Authors' Affiliation:
1. Chemistry Department, College of Science, Al-Qadisiyah University - Iraq
2. Biochemistry Department, College of Medicine, Al-Qadisiyah University - Iraq

***Corresponding Author:**
Ferdous A Jabir
Email: ferdous.jabir@qu.edu.iq

How to Cite:
Hatem BA, Jabir FA (2025). Ferroportin Gene Polymorphism, Ferritin, and TIBC Levels Associated with the Severity of COVID-19 among patients: A Sequencing Analysis. Adv. Life. Sci. 12(4): 811-816.

Keywords:
COVID-19; Ferroportin; ferritin; polymorphism; Sequencing; TIBC

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Ferroportin Gene Polymorphism, Ferritin, and TIBC Levels Associated with the Severity of COVID-19 among patients: A Sequencing Analysis

Ban Adnan Hatem[1], Ferdous A Jabir[2]*

Abstract

Background: SARS-CoV-2 may also be termed a high-load virus due to its rapid spread in the bloodstream. In vitro studies verify that iron chelators can inhibit the virus, indicating iron's key component in viral replication. What's more, increased apoptosis (ferroptosis) due to intracellular accretion of iron has been discovered in biopsy specimens of COVID-19 patients. Unlike harsh pneumonitis, COVID-19 does not demonstrate a hepcidin-initiated accretion of iron at foci of infection, suggesting a different iron metabolism profile.

Methods: This work explored COVID-19 risk and severity according to Ferroportin gene polymorphisms, SNPs rs3811621 and rs10202029. Infected and recovered individuals' samples were utilized for genotypic analysis to observe possible genetic associations.

Result: There were no notable associations of SNP rs3811621 and SNP rs10202029 both with susceptibility to COVID-19, and harshness of the disease. Frequencies of genotype between patient and recovery groups did not differ. Ferritin values, however, were significantly different between mild, severe, and recovery cases. Total iron-binding capacity (TIBC) was significantly lower in recovery subjects than in moderate or severe disease subjects, a predictable and significant finding.

Conclusion: Ferroportin polymorphisms (rs3811621 and rs10202029) do not appear to influence COVID-19 susceptibility and severity. In spite of that, variations in ferritin and TIBC across different patient populations demonstrate their significance as biomarkers in defining iron metabolism during and after infection.

Introduction

Iron transport from the inside to the outside of the cell is achieved by a protein known as Ferroportin (Fpn), which is the known protein for this purpose [1]. Human Ferroportin is encoded by SLC40A1 gene [2].

Following the absorption of iron from food in the gastrointestinal tract, Ferroportin transports iron from intestinal cells into the bloodstream. Fpn also participates in iron effluxes from macrophages in the spleen and liver [3].

The aforementioned functions of Ferroportin can all be controlled by hepcidin, which is a liver protein. A reduction in Iron plasma concentration is achieved when hepcidin binds to Fpn by a reduction in iron-efflux activity. Therefore, interaction between hepcidin and Fpn governs systemic iron homeostasis [4].

Hepcidin concentrations are usually associated with iron trapping in SD3 enterocytes, macrophages, and hepatocytes [5]. It is transcriptionally produced by HAMP gene expression. Several factors play a role in regulating its expression, positively represented by an increment of iron overload, inflammation, decreased iron-deficient states, and hypoxia [6]

Total iron-binding capacity (TIBC) refers to the highest amount of transferrin (TRF)-binding iron that will attach to transferrin, which is the most common protein that carries iron in plasma or serum when in the bloodstream. It's frequently tested together with serum iron to better establish one's diagnosis of a deficit or surplus of iron [7]. Dividing the results of both tests, transferrin saturation is calculated—a measure that's considered more precise than either concentration of iron or TIBC alone when one's status for iron is being measured. Abnormal TIBC levels can indicate a range of diseases, including hereditary hemochromatosis, various anemias, pregnancy, liver disease, and malnutrition [8].

The unsaturated iron binding capacity (UIBC) is used to measure the unsaturated portion of serum transferrin. Unlike transferrin saturation, which requires two analytical steps and may be prone to laboratory errors, UIBC is a readily automatable one-step chemical test. UIBC has been suggested as a low-cost screening assay for iron overload and may also be useful for population screening for hemochromatosis [9].

The purpose of this study is to investigate the relationship between the severity of COVID-19 and the levels of Ferroportin, ferritin, and TIBC, and to explore the presence of Ferroportin gene polymorphism in the study groups [10].

Methods

Declarations

The ethical committee of Al-Qadisiyah University approved this study after the submission of written consents from the participating individuals. The study included one hundred and fifteen patients diagnosed with COVID-19 at AL-Dewania hospital in Iraq between February 2022 and May 2022. The design of the study is a case-control study.

Collection and storage of samples

Each subject provided a blood sample of 5 milliliters drawn from a vein and divided into two separate test tubes. An EDTA tube was filled with one milliliter of the blood for the analysis of gene polymorphism. A Gel tube was filled with the remaining 4 milliliters of the collected blood sample for biochemical analysis. Blood sample in that gel tube was spun at 3000 ×g for 10 minutes to separate the serum, which was portioned into three individual Eppendorf tubes and stored at -20 °C until they were tested. Meanwhile, relevant investigations were conducted immediately on whole blood.

Sample preparation for polymorphism

Each frozen blood sample was thawed, centrifuged; genomic DNA was then extracted directly using the New England Biolabs® Inc. Blood DNA extraction kit. DNA concentration was measured using Qubit 4(Invitrogen, USA).

Hepcidin genotyping

The association between the Ferroportin gene and COVID-19 was investigated; molecular studies were conducted using polymorphism sequencing techniques. The PCR reaction was performed under optimal conditions for this gene, and PCR products of more than 40µl from each sample were sent to Macrogen-Korea for sequencing using the Sanger method to identify any single-nucleotide polymorphisms within the promoter region. The sequence FASTA files were analyzed using Desktop Geneious Bioinformatics Suite and aligned with Ferroportin gene's RefSeq identifier, accession number: NG_009027.

Determination of Ferritin Levels by ELISA

A Human Serum Ferritin (SF) ELISA kit, bought from SunLong, China, was employed to assess the blood sample concentration of Ferroportin by following the enzyme-linked immunosorbent assay method.

TIBC analysis and measurement

TIBC was estimated by an automated analyzer (Aboot), which also estimated serum iron and UIBC.

Statistical analysis

The data obtained were analyzed using SPSS ver. 23.1 software (SPSS, Chicago, IL, USA). Relationships between categorical variables were evaluated using

Pearson's χ^2-test. The Hardy-Weinberg equilibrium of alleles at individual loci was evaluated using 2 statistics. Logistic regression was performed using the same software to compute odds ratios (OR) with Cornfield 95% confidence intervals (CIs).

Participants and Sample Collection

Sampling of biological samples took place between February and May of 2022. Participants were separated into 3 distinct classifications:

Group 1 (G1): People previously infected with COVID-19 but now recovered from it.

Group 2 (G2): Individuals infected with COVID-19 currently, who were characterized by doctors as mild cases.

Group 3 (G3): Individuals who were already infected with COVID-19 and were characterized as severe cases by doctors.

A total of 115 samples were yielded. Among them, 50 subjects had recovered their complete health status after COVID-19 for over three months and had no lingering post-infection symptoms.

Other 100 cases were included under mild and severe conditions. Outpatients were selected for mild cases after they were diagnosed based on a combination of clinical markers, including D-dimer, CRP, antigen/antibody tests, and chest CT scans. Severe cases were selected from Al-Dewania Hospital's Respiratory Care Unit (RCU).

Eligibility Criteria

Inclusion in the study included confirmation of COVID-19 infection by PCR, CT imaging, or other appropriate biochemical assessments.

Exclusion Criteria

Patients diagnosed with iron deficiency anemia, thalassemia, chronic or autoimmune diseases such as rheumatoid arthritis, renal diseases, severe liver diseases, and cancer. Other cases included individuals lacking laboratory-confirmed COVID-19 infection despite clinical suspicion and had underlying health issues, including cardiovascular disease, diabetes, and hypertension.

Results

Ferritin protein expression

Statistical significance in ferritin values did not exist when mean values were compared across different case groups. Specifically, when mild and severe cases, mild and recovered cases, and severe and recovered cases were compared, they were all highly significant ($p < 0.0001$). All of these differences are graphically represented in Figure 1.

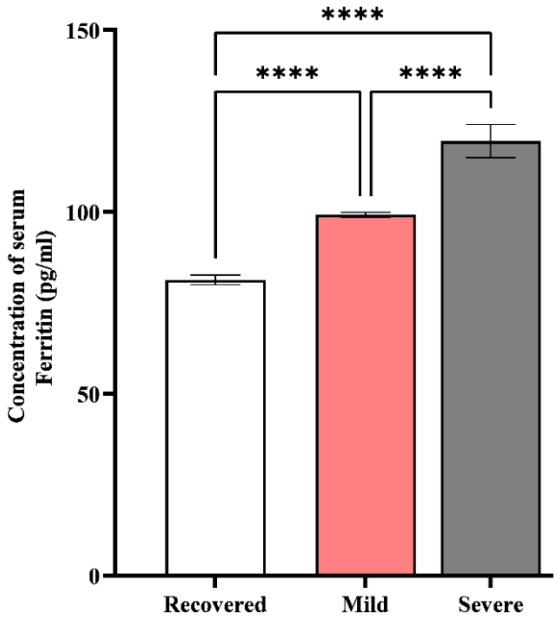

Figure 1: Assessment of COVID-19 patients' serum ferritin levels in ng/ml.
This figure illustrates the comparison of ferritin mean values among mild cases, severe cases, and recovered individuals. Significant differences were observed between 1st and 2nd group ($p < 0.0001$), 1st and 3rd group ($p < 0.0001$), and 2nd and 3rd group ($p < 0.0001$). The figure highlights the distinct variations in ferritin levels across the three groups. *** indicated a p-value of ≤ 0.0001 and **** indicated a p-value of ≤ 0.0001

There were significantly high increases ($p < 0.0001$) in serum levels of ferritin when severe and mild cases were compared in cases of COVID-19. Also, there were significant increases ($p < 0.0001$) in levels of ferritin in two groups when compared to those that had recovered.

Total Iron-Binding Capacity (TIBC)

Analysis of TIBC also revealed highly significant difference in mean values between groups, specifically severe and mild, recovered and mild, and severe and recovered groups, all of which had corresponding p-values less than 0.0001, as demonstrated in Figure 2.

The study found a noteworthy reduction (p-value < 0.0001) in the serum TIBC concentration between mild and severe cases of COVID-19 patients, and a significantly higher concentration (p-value < 0.0001) in recovered patients.

DNA Detection

To assess and confirm the accuracy of the DNA extraction process, electrophoresis was performed on all the extracted DNA samples. Figure 3 displays the DNA bands for a selection of the analyzed samples.

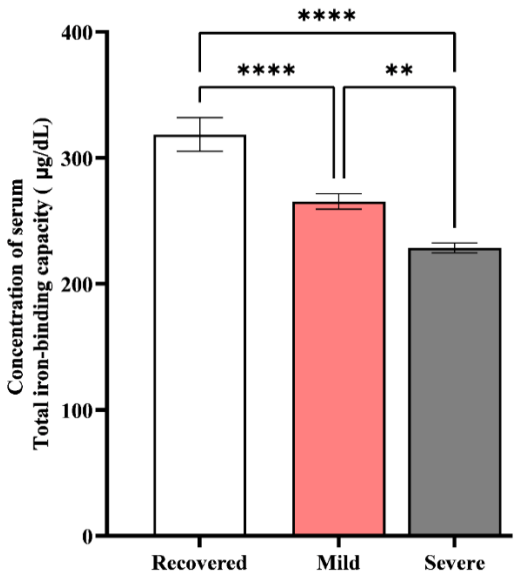

Figure 2: Estimation of serum concentrations of TIBC concerning COVID-19 patients.
This figure illustrates the comparison of total iron-binding capacity (TIBC) mean values across mild cases, severe cases, and recovered individuals. The figure highlights the distinct variations in TIBC levels among the three groups

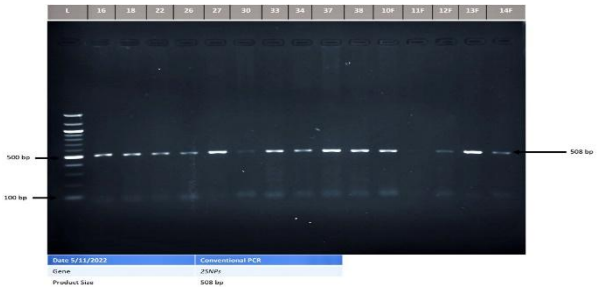

Figure 3: Agarose gel electrophoresis for detection of Ferroportin gene.
This figure depicts a 1.5% agarose gel electrophoresis stained with RedSafe® and run at 80V. The gel shows the detection of the Ferroportin gene across lanes 1 to 15, with a PCR product size of 420 bp. Lane 'L' represents the DNA ladder with 100 bp step markers, which serves as a molecular size reference. This visualization confirms the successful amplification of the Ferroportin gene

Sequence Analysis of Polymorphism of the Ferroportin gene

The initial method of iron absorption occurs in two forms: ferrous (Fe^{2+}) or ferric (Fe^{3+}) iron, which is absorbed by intestinal enterocytes using divalent metal transporter (DMT1). The absorbed iron is then released into the bloodstream from the intestinal enterocytes using Ferroportin-mediated transferrin-bound iron (TBI) [11]. There are only two known cellular iron release mechanisms, in contrast to the numerous pathways of cellular iron uptake. Typically, iron is released from a cell through Ferroportin (Fpn), a membrane-bound iron exporter regulated by hepcidin produced by hepatocytes [12]. This study used new primers to amplify the region where polymorphism is present. The PCR product size for this region was 508 bp, and 100 samples (70 from patients and 30 from recovered individuals) were sent for sequencing with primer 2SNP-NF CAGGGTTTTCGTGAGATTAAG and the reverse primer 2SNP-NR CCCTCAAAGAACCAGAATCAA. The results were analyzed as mentioned in the methods, and a quality check of each sequence was conducted.

Genetic variation (polymorphism) frequency in the Ferroportin gene (rs3811621) among two groups is shown in Table 1: a group of recovered individuals (G1) and a group of patients (G2).

Table 1 presents the number and percentage of individuals with each genotype (CC, GG, and GC) and allele (C allele, G allele) in each group.

The main findings of Table 1 are that the frequency of the C allele is significantly higher in the recovered group (77.3%) than in the patient group (89.3%) (p = 0.047), with an OR of 0.406 (95% CI: 0.163-1.007).

Genotypes GG, CC, and GC showed no noteworthy variances in their frequencies between the two groups. The odds ratios for each genotype comparison (CC vs GG, CC vs GC, and GG vs GC) are not statistically significant.

In summary, this table suggests that there may be a difference in the frequency of the C allele of the rs3811621 polymorphism between recovered individuals and patients, but there is no significant difference in the frequency of each genotype. However, it is important to note that this is just one study, and further research is needed to confirm these findings.

Ferroportin gene rs3811621	G1 recovered N = 22(%)	G2 patients N = 61(%)	χ^2	p-value	OR (95%CI)	p-value
CC GG GC	14(63.6) 2(9.1) 6(27.3)	49(80.3) 1(1.6) 11(18.1)	3.751	0.153	1.0^{ref} (1.0^{ref}) 0.134(0.012-1.694) 0.524(0.164-1.669)	0.79 0.269
C allele G allele	34(77.3) 10(22.7)	109(89.3) 13(10.7)	3.948	0.047	1.0^{ref} (1.0^{ref}) 0.406(0.163-1.007)	N.A
CC GG&GC	14(63.6) 8(36.4)	49(80.3) 12(19.7)	2.469	0.117	1.0^{ref} (1.0^{ref}) 0.429(0.146-1.254)	N.A
GG CC&GC	2(9.1) 20(90.9)	1(1.6) 60(98.4)	2.577	0.108	1.0^{ref} (1.0^{ref}) 0.167(0.014-1.938)	N.A

Table 1: FNP (rs3811621) polymorphism occurrence in groups of recovered and sick individuals.
Table 1 shows the frequency distribution of the Ferroportin (FPN) gene polymorphism rs3811621 among recovered individuals (G1, N = 22) and patients (G2, N = 61). The table presents the genotypic distribution of CC, GC, and GG genotypes, as well as the allelic frequency of C and G alleles for both groups. Chi-square ($\chi 2$) values, odds ratios (OR) with 95% confidence intervals (CI), and p-values are included to assess the statistical significance of differences between groups. The comparisons are made between CC vs. GG&GC, CC&GC vs. GG, and C vs. G alleles to evaluate the potential association of the rs3811621 polymorphism with patient status. N.A = not available

Ferroportin gene rs10202029	G1 Control N = 22(%)	G2 Patients N = 85(%)	χ^2	p-value	OR (95%CI)	p-value
GG AA GA	19(86.4) 00(0.00) 3(13.6)	73(85.9) 2(2.4) 10(11.7)	0.569	0.753	1.0^{ref} (1.0^{ref}) 1.027(0.990-1.067) 1.153(0.288-4.607)	0.472 0.841
G allele A allele	41(93.2) 3(6.8)	156(91.8) 14(8.2)	0.096	0.757	1.0^{ref} (1.0^{ref}) 0.815(0.224-2.972)	N.A
GG AA&GA	19(86.4) 3(13.6)	73(85.9) 12(14.1)	0.003	0.954	1.0^{ref} (1.0^{ref}) 0.961(0.246-3.750)	N.A
AA GG&GA	0(0.00) 22(100)	2(2.4) 83(97.6)	0.528	0.468	1.0^{ref} (1.0^{ref}) 0.167(0.014-1.938)	N.A

Table 2: FPN (rs10202029) polymorphism occurrence in groups of recovered and sick patients.

Table 2 presents the distribution and frequency of the Ferroportin (FPN) gene polymorphism rs10202029 among recovered individuals (G1, N = 22) and patients (G2, N = 85). The genotypic distribution of GG, GA, and AA genotypes is displayed alongside the allelic frequency of G and A alleles for both groups. Chi-square ($\chi 2$) values, odds ratios (OR) with 95% confidence intervals (CI), and p-values are provided to highlight the statistical significance of differences between the groups. Comparisons are made between GG vs. AA&GA, GG&GA vs. AA, and G vs. A alleles to assess the association of the rs10202029 polymorphism with patient status. N.A = not available

These data represent a comparison of the frequency of a specific genetic variation in the Ferroportin gene (rs10202029) between two groups: a control group of 22 individuals (G1) and a group of 85 patients (G2). The genotypes of the individuals are classified as GG, AA, or GA. The frequency of each genotype is compared between the two groups (Table 2).

The results showed that the frequency of the GG, AA, and GA genotypes is similar between the control and patient groups. The values $\chi^2 = 0.569$ and $p = 0.472$ indicate that there is no significant difference between the groups.

The OR for the AA genotype is 1.027, indicating a very slight increase in the odds of being a patient for individuals with this genotype compared to the reference GG genotype. However, the 95% CI includes 1.0, indicating that this result is not statistically significant ($p = 0.841$).

The frequency of the G and A alleles is also similar between the two groups. The values $\chi^2 = 0.096$ and $p = 0.757$ indicate that there is no significant difference.

The OR for the combined AA and GA genotypes compared to the GG genotype is 0.961, indicating a slight decrease in the odds of being a patient for individuals with these genotypes. However, the 95% CI includes 1.0, and $p = 0.954$ suggests a similar output as that of AA genotype.

The OR for the combined GG and GA genotypes compared to the AA genotype is 0.167, indicating a very large decrease in the odds of being a patient for individuals with these genotypes. Similar output like that of AA and GA genotype was also suggested here by 95% CI value, which is 1.0 and $p = 0.468$.

Overall, the data propose a non-significant alteration in the incidence of rs10202029 polymorphism in Ferroportin gene between control and patient groups. While there are some slight differences in the odds of being a patient associated with certain genotypes, these differences are not statistically significant.

Discussion

Ferritin protein expression

Ferritin is the principal storage molecule for iron in its ferric (Fe^{3+}) form, which can store 4,500 atoms of iron in its molecule [13]. High serum ferritin most frequently occurs in systemic inflammatory processes. During them, pro-inflammatory cytokines, including interleukin-6 (IL-6), stimulate production of ferritin and of hepcidin (a major hormone in negative feedback regulation of iron). Hepcidin acts by sequestering iron in macrophages and enterocytes (intestinal cells), resulting in accrual of ferritin in cells and inhibition of release of iron into bloodstream [14].

Therefore, we hypothesize that elevated serum ferritin levels due to hyperinflammation caused by COVID-19 may indicate a harmful loop of events where increased ferritin levels could result in additional tissue harm. Excessive iron within cells reacts with oxygen and creates reactive oxygen species [13,15]. This could significantly contribute to oxidative damage of cellular parts in various organs such as the lungs, liver, kidneys, and heart. Various studies support the connection between increased ferritin levels and different inflammatory conditions, such as cardiovascular events [16].

Total Iron-Binding Capacity (TIBC)

Iron, UIBC, and TIBC levels were found to be lower in patients than in recovered individuals. Anemia, characterized by a hemoglobin level below 13.0 g/dl, is often caused by iron deficiency, which may occur due to its role in inhibiting infectious and inflammatory responses or in skeletal muscle weakness, which can reduce breathing capacity [17]. COVID-19 is associated with anemia of inflammation. A direct relationship was observed between iron and the two markers, UIBC and TIBC, with a lack of iron leading to a lack of these markers [18]. This study suggests that the low iron levels in COVID-19 patients may contribute to their respiratory deterioration and even death.

Ferroportin (DNA Detection)

The analysis of rs3811621 and rs10202029 polymorphisms of the Ferroportin gene revealed differences in genotype and allele frequencies between recovered individuals and patients. The frequency of the C allele for rs3811621 was higher in the recovered group, which may suggest a potential association with disease recovery. Further investigation is required to validate these findings and to explore the functional impact of these polymorphisms on disease severity and recovery.

Acknowledgement

The authors would like to extend their sincere gratitude and appreciation to the hardworking and dedicated staff of Al-Dewania Teaching Hospital for their valuable contributions to this study. The tireless efforts and unwavering commitment of the hospital's healthcare workers to providing exceptional care to patients have made this research possible.

Author Contributions

Ban Adnan Hatem and Ferdous A Jabir both equally contributed to the design, sampling, data collection and analysis, writing, and finalization of the research article.

Competing Interest

There are no conflicts of interest in this study.

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