Original Article

Investigating the Hepcidin Gene Polymorphisms in COVID-19-Associated Mucormycosis Susceptibility: A Clinical-Laboratory Study

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What's Known

- The emergence of COVID-19-associated mucormycosis, which is characterized by dysregulated inflammation and iron metabolism, exacerbated the prognosis of affected patients.
- Studies have shown that one of the important genes involved in dysregulated inflammation and iron metabolism is hepcidin.

What's New

- The results revealed the potential of some factors and genotypes in mucormycosis susceptibility. In contrast to patients without mucormycosis, patients with mucormycosis did not display SNP442GA and SNP335GT genotypes.
- Increased levels of urea, aspartate aminotransferase, lactate dehydrogenase, and increased ratio of polymorphonuclear neutrophils to lymphocytes are associated with a decreased risk of mucormycosis.

Abstract

Background: Following the coronavirus disease 2019 outbreak (COVID-19), it became a worrisome health burden worldwide. COVID-19-associated mucormycosis emergence, characterized by dysregulated inflammation and iron metabolism, exacerbated the prognosis of affected patients. Given the significance of hepcidin in regulating inflammation and iron metabolism, this study investigated the significance of hepcidin single nucleotide polymorphisms (SNP) in COVID-19-associated mucormycosis development, along with the association between the clinical and laboratory factors and COVID-19-associated mucormycosis.

Methods: From September 2021 to November 2021, COVID-19 patients with and without mucormycosis were enrolled in this cross-sectional study. Their medical records and laboratory results were investigated. SNP genotyping was performed using Sanger sequencing. Hardy-Weinberg Equilibrium, Pearson's Chi square, and student *t* test were used for analyzing the data using SPSS software version 25. $P<0.05$ was regarded as statistically significant.

Results: Here, 110 COVID-19 patients with and without mucormycosis were investigated. Elevated levels of urea, aspartate aminotransferase, lactate dehydrogenase, and increased ratio of polymorphonuclear neutrophil to lymphocytes were associated with decreased risk of COVID-19-associated mucormycosis in patients (all $P<0.05$). Moreover, diabetes mellitus increased the risk of mucormycosis ($P=0.028$). In contrast to patients without mucormycosis, patients with mucormycosis did not display 442 GA and SNP335 GT genotypes. Unlike patients without mucormycosis, none of the patients with mucormycosis had SNP442 GA and SNP335 GT genotypes. Regarding SNP 443 C>T, and the combination of SNPs 582 A>G and 443 C>T, CC genotype and AA+CC genotypes were associated with increased lactate dehydrogenase levels in COVID-19 patients, respectively.

Conclusion: Regarding SNP 443 C>T, the CC genotype was associated with increased lactate dehydrogenase levels in COVID-19 patients. In terms of SNP 582 A>G and SNP 443 C>T, COVID-19 patients with AA+CC genotypes had higher levels of LDH. None of the patients with mucormycosis had SNP442 GA and SNP335 GT genotypes.

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) led to the coronavirus disease 2019 (COVID-19) outbreak in 2019.[1] The initial pneumonia-resembling symptoms of COVID-19 can lead to the development of hypoxia, acute respiratory disease syndrome (ARDS), and systemic inflammatory response syndrome in some patients.[2]

Inflammation is highly implicated in developing severe clinical manifestations of COVID-19.[3] The dysregulation of pro-inflammatory cytokines and chemokines, *e.g.*, IL-6, pave the way for cytokine storm and multi-organ failure in affected patients.[4] Aside from the systemic inflammatory response syndrome, the development of mucormycosis has been another worrisome concern in patients.[5] Mucormycosis is an opportunistic fungal infection that highly affects the blood vessels; low pH, hyperglycemia, and iron metabolism dysregulation have been identified for the emergence of mucormycosis.[6] *Rhizopus oryzae* and *Mucor circinelloides* are among the causative fungal organisms for mucormycosis. Fungal spores inhalation is the main route for developing mucormycosis in immunocompromised patients. It has been reported that immunocompromised patients, such as patients with organ transplants, leukemia, and those on chronic use of steroid-based agents, are more prone to develop mucormycosis.[6] Besides the hyperglycemic complication of glucocorticoid administration for treating COVID-19 patients, COVID-19-related tissue injury and dysregulated inflammation can increase free iron and ferritin levels, facilitating mucormycosis development.[7] Therefore, dysregulated inflammation and iron metabolism have substantial roles in developing mucormycosis in COVID-19 patients.

Hepcidin, encoded by hepcidin antimicrobial peptide (*HAMP*), is well-known for its significant role in regulating iron homeostasis. Increased iron levels upregulate hepcidin expression, leading to decreased iron levels.[8] Hepcidin is produced from the hepatocytes and acts as a receptor of ferroportin, which is highly expressed in enterocytes and macrophages. The hepcidin-mediated signaling pathways block the iron absorption and iron export in macrophages.[9] Besides, increased levels of hepcidin reported in inflammation and infection, likely reduce iron availability for the microorganisms. IL-6-mediated hepcidin expression can be the underlying mechanism for hepcidin upregulation in inflammations and infections.[10] Considering the substantial role of dysregulated inflammation in the pathogenicity of COVID-19, it has been reported that hepcidin is considerably elevated in non-survivor COVID-19 patients admitted to ICU compared to survivor ones.[11] Recently, it was shown that *Rhizopus arrhizus* var. delemar, which is responsible for mucormycosis, can secrete peptides that regulate hepcidin expression in blood-derived monocytic macrophages.[12] Although hepcidin has a significant role in iron hemostasis and inflammation, and inflammation and iron dysregulation have been implicated in COVID-19 pathogenesis and COVID-19-associated mucormycosis development, to the best of our knowledge there is no study to investigate the role of hepcidin polymorphisms in COVID-19-associated mucormycosis development.

Genetic footprint has been identified in most human conditions. Different expression patterns have been implicated in developing and progressing human diseases such as COVID-19.[13] In this regard, polymorphism is highlighted as one of the main culprits for altering the expression of genes. Specific nucleotide changes in the promotor of genes can affect its expression, leading to the altered function of that gene product.[14] It has been reported that single-nucleotide polymorphism (SNPs) of *HAMP* can also alter iron metabolism.[15] The SNP582 A>G, SNP 443 C>T, SNP 442 G>A, SNP 335 G>T, and SNP 153 C>T are the polymorphisms of the promotor region of the *HAMP* gene, and the number mentioned in SNP names refer to their exact location in the promotor. SNP582 A>G is one of the well-studied polymorphisms of *HAMP*, wherein the presence of G allele decreases hepcidin expression.[16] SNP 153 C>T variant is located in a bone morphogenetic protein (BMP)-responsive element and reduces basal hepcidin gene expression by disrupting its response to BMPs and IL-6.[17] SNP 443 C>T, SNP 442 G>A, and SNP 335 G>T were later included in the study when sequencing data revealed heterozygosity in those loci.

Despite the impact of SNPs on hepcidin expression and the significant role of hepcidin in COVID-19 pathogenesis and COVID-19-associated mucormycosis development, there is no study investigating the effect of *HAMP* SNPs on COVID-19-associated mucormycosis development.

The current study investigated the clinical and laboratory characteristics of COVID-19 patients who developed COVID-19-associated mucormycosis. Moreover, the significance of hepcidin polymorphisms in the clinical and laboratory features of COVID-19-associated mucormycosis was investigated.

Patients and Methods

Study Participants

From September 2021 to November 2021, COVID-19 patients with and without mucormycosis who were admitted at Emam Reza and Sina Hospitals, the teaching Hospitals affiliated with Tabriz University of Medical Sciences (Tabriz, Iran), were enrolled in this cross-sectional study. Based on the statistical power analysis, a sample size of 110 participants was selected (error margin of 5% with a 95% confidence interval). As inclusion criteria, patients who were admitted to the hospitals due to COVID-19-associated mucormycosis during the delta wave were included as cases, and people who had a history of hospitalization due to COVID-19 without mucormycosis were included in the study as the control group. Those who had previous history of mucormycosis or were not positive for SARS-CoV-2 were excluded. This study was performed after approval by the Ethics Committee of Tabriz University of Medical Sciences (Ethical approval no: IR.TBZMED.REC.1400.1086). Besides, the protocol of this study was performed following the Declaration of Helsinki. The included patients' demographical, clinical, and laboratory characteristics were collected for further analyses.

Hepcidin Gene Polymorphisms

Following written informed consent, 5 mL of peripheral venous blood was collected from each patient into separate EDTA-coated blood collection tubes. Following red blood cell lysis and centrifuging, the DNA of white blood cells was extracted using the salting out method.[18] The extracted DNA was preserved in an RNAse and DNAase-free microtube at -20 °C. The quality and quantity of the extracted DNA were assessed using agarose gel (2%) and spectrophotometry. Using the Gene Runner, the related primers for amplifying a fragment containing SNP 582 A>G, SNP 443 C>T, SNP 442 G>A, SNP 335 G>T, and SNP 153 C>T polymorphisms were designed, and the specificity of the primers was studied using the NCBI website (https://www.ncbi.nlm.nih.gov/). The fragment was amplified using the Polymerase Chain Reaction (PCR) technique. The primers for the amplification of the 666 bp target sequence containing the polymorphic regions of interest were forward 5′-TATTACTGCTGTCATTTATGGC-3′ and 5′-reverse TCTGTCTGGCTGTCCCACT-3′. PCR on the target fragment was conducted in a reaction mixture including 12.5 µL Taq DNA Polymerase Master Mix RED (Ampliqon, Denmark), 1 µL of each mentioned primer (10 pmol), 1 µL template DNA (30-60 ng), and finally 9.5 µL sterile distilled H_2O.

PCR was carried out in a thermal cycler (Sensoquest, GmbH, Germany) after setting the following cycling program: an initial denaturation for 5 min at 94 °C followed by 35 cycles of denaturation for 35 sec at 94 °C, primer annealing for 30 sec at 60 °C, extension for 35 sec at 72 °C, and a final extension for 7 min at 72 °C. Then, 5 µL of each PCR product was loaded on a 2% agarose gel to evaluate the PCR reaction. To obtain high-quality sequencing results, PCR reactions should be specific. A 50 bp DNA ladder, as a molecular size marker (Fermentas, USA), was also loaded into a well of gel. The amplified fragments were sequenced using the Sanger method (Bio Magic Gene (BMG) Co., China) to identify the SNPs. The sequencing data were interpreted by Chromas 2.6 software (Nucleics Pty Ltd., Australia).

Statistical Analysis

After SNP genotyping, the statistical analysis was conducted using SPSS software V.25 (SPSS Inc., Chicago, USA). The Hardy-Weinberg Equilibrium (HWE) was applied to assess the genotype distributions in the case and control populations using an HWE calculator, available at http://www.oege.org/software/hwe-mr-calc.shtml. Pearson's Chi square or Fisher exact test was used to study the possible associations between COVID-19-associated mucormycosis with these polymorphisms and patients' clinical and laboratory characteristics. The odds ratio was calculated using the binary logistic regression, and the 95% CI was calculated. The Student *t* test was used to compare the two groups, and the normality of data distribution was studied via the Kolmogorov-Smirnov or Shapiro-Wilk tests. Levene's test was used to study the equality of variances. A $P<0.05$ was considered statistically significant.

Results

The Association between Clinical and Laboratory Findings and COVID-19-Associated Mucormycosis

Table 1 shows the associations between the studied clinical and laboratory variables of included patients with the development of mucormycosis. It was found that increased levels of urea, aspartate aminotransferase (AST), lactate dehydrogenase (LDH), iron, and elevated ratio of polymorphonuclear neutrophil (PMN) to lymphocytes were associated with decreased risk of COVID-19-associated mucormycosis in COVID-19 patients. Table 2 demonstrates

Table 1: Laboratory characteristics of patients and estimated P values for COVID-19 and mucormycosis association with laboratory status

Laboratory characteristics	Total (n=110) mean±SEM	Patients without mucormycosis# (n=60) mean±SEM	Patients with mucormycosis (n=50) mean±SEM	P value	(95% CI)	OR (95% CI)
WBC	(110) 9217.09±470.18	(60) 9925±776.71	(50) 8367.6±428.01	0.419	0.37-0.557	1 (1-1)
Hb	(110) 12.15±0.26	(60) 12.8±0.39	(50) 11.4±0.29	0.005*	0.48-2.41	0.807 (0.69-0.94)
Plt	(110) 239163.6±9724.99	(60) 233383.3±15042.45	(50) 246100±11580.01	0.504	-5.6-4.12	1 (1-1)
MCV	(92) 85.49±0.99	(46) 85.02±1.39	(46) 85.95±1.43	0.225	0.133-0.285	1.011 (0.97-1.06)
RBC	(91) 4.09±0.084	(45) 4.24±0.14	(46) 3.94±0.09	0.083	-0.039-0.637	0.625 (0.37-1.07)
Cr	(107) 1.77±0.19	(60) 2.11±0.39	(50) 1.36±0.14	0.104	0.04-0.156	0.719 (0.51-1.03)
LDH	(75) 547.42±35.63	(60) 608.45±49.83	(50) 432.42±32.38	0.007*	0.000-0.27	0.996 (0.993-0.999)
Ferritin	(15) 515±84.16	(7) 521.57±124.12	(8) 509.87±122.47	0.948	0.48-2.41	1 (0.997-1.003)
Iron	(8) 83.88±11.71	(5) 101.6±11.94	(3) 54.33±10.33	0.036*	-4.17-90.36	0.85 (0.61-1.19)
Alt	(71) 37.14±8.53	(37) 48.86±16.04	(34) 24.38±2.7	0.333	0.291-0.473	0.986 (0.96-1.003)
AST	(70) 30±3.81	(37) 35.97±6.71	(33) 23.3±2.64	0.031*	0.007-0.84	0.969 (0.94-1.004)
ESR	(46) 49.4±4.89	(27) 47.22±5.84	(19) 52.53±8.6	0.639	0.546-0.726	1.005 (0.99-1.02)
CRP	(76) 60.16±7.46	(46) 61.18±9.25	(30) 58.6±12.68	0.857	0.8-0.228	0.999 (0.992-1.007)
Polymorpho nuclear leukocytes	(62) 73.78±1.7	(26) 78.52±2.4	(36) 70.37±2.22	0.017*	1.52-14.78	0.95 (0.909-0.993)
Lymphocytes	(68) 19.93±1.44	(31) 15.98±1.86	(37) 23.24±2.01	0.01*	-12.73-1.79	1.06 (1.01-1.11)
HCT	(81) 35.21±0.76	(37) 36.19±1.34	(44) 34.38±0.82	0.252	-1.32-4.95	0.961 (0.91-1.02)
Urea	(100) 53.9±4.07	(54) 62.67±6.51	(46) 43.61±4.03	0.015*	3.84-34.28	0.985 (0.97-0.99)

*P value is significant at P<0.05. All Ps were calculated by *t* test. #In all OR logistic regression models, patients without mucormycosis were considered as a reference. WBC: White blood cell; Hb: Hemoglobin; Plt: Platelet; MCV: Mean corpuscular volume; RBC: Red blood cell; Cr: Creatinine; LDH: Lactate dehydrogenase; ALT: Alanine transaminase; AST: Aspartate transferase; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; HCT: Hematocrit

the association between the studied clinical and laboratory variables of COVID-19 patients with mucormycosis. Our results showed that diabetes mellitus significantly increases the risk of mucormycosis development in COVID-19 patients.

The Frequency of HAMP Genotypes in COVID-19 Patients with and without Mucormycosis

The sequencing diagrams of the polymorphisms are shown in figure 1. Based on the HWE test, the genotype frequency distribution of the control and case individuals followed the Hardy-Weinberg equilibrium for the studied polymorphisms (table 3).

Afterward, the frequency of hepcidin gene polymorphisms in COVID-19 patients with and without mucormycosis was studied (table 3). Significant differences were observed in the frequency of SNP335 G>T in COVID-19 patients with mucormycosis compared to those without mucormycosis. Some COVID-19 patients without mucormycosis had the T allele, which seems to reduce the risk of mucormycosis. However, since there was no T allele in COVID-19 patients with mucormycosis, we could not compute OR value. Indeed, none of our COVID-19 patients with mucormycosis were GT in terms of SNP335; while three COVID-19 patients without mucormycosis were GT. There were no significant differences between the studied polymorphisms of SNP582 A>G, SNP443 C>T, SNP442 G>A, and the risk of mucormycosis. Since all included patients were CC regarding SNP 153C>T, the related statistical analysis was not performed. Although the SNP442 GA genotype was found in one of our COVID-19 patients without mucormycosis, none of our COVID-19 patients with mucormycosis had this genotype.

Table 2: Clinical characteristics of patients and estimated P values for COVID-19 and mucormycosis association with clinical status

Character (n)	Groups	Patients without mucormycosis n (%)	Patients with mucormycosis n (%)	P value	Ref OR (95% CI)
Sex (110)	Female	25 (41.7)	24 (48)	0.506	1
	Male	35 (58.3)	26 (52)		0.807 (0.29-2.34)
Marital status (77)	Single	5 (16.7)	5 (10.6)	0.44	1
	Married	25 (83.3)	42 (89.4)		1.68 (0.44-6.38)
PMH (75)	Yes	26 (86.7)	35 (77.8)	0.333	1
	No	4 (13.3)	10 (22.2)		0.146 (0.03-0.69)
DM (74)	No	18 (60.0)	15 (34.1)	0.028*	1
	Yes	12 (40.0)	29 (65.9)		6.86 (2.01-23.4)
Age (years)	(108) 58.16±1.44 Range (22-95)	(60) 57.5±2.21#	(48) 58.68±1.72	0.673	1# 0.995 (0.97-1.02)

*P value is significant at P<0.05. All Ps were calculated by *t* test. PMH: Past medical history, comorbidities other than DM including hypertension, hyperlipidemia, cerebrovascular accident, ischemic heart disease, chronic renal failure, hyperthyroidism, and cancer; DM: Diabetes mellitus; Ref: Reference line

Table 3: Frequency of genotypes in COVID-19 patients with and without mucormycosis performed by HWE analysis

SNP582 A>G							
Group	**AA n (%)**	**AG n (%)**	**GG n (%)**	**Total**	**95% CI**	**P value**	**HWE P value**
MUCOR	28 (57.10)	19 (38.80)	2 (4.10)	49 (100)	0.807 -0.821	0.771	0.58
COVID	32 (57.10)	23 (41.10)	1 (1.80)	56 (100)			0.17
Total	60 (57.1)	42 (40)	3 (2.9)	105 (100)			0.17
SNP443 C>T							
Group	**CC n (%)**	**CT n (%)**	**Total**		**95% CI**	**P value**	**HWE P value**
MUCOR	43 (87.8)	6 (12.2)	49 (100)		0.724-19.611	0.90	0.21
COVID	54 (96.4)	2 (3.6)	56 (100)				0.89
Total	97 (92.1)	8 (7.9)	105 (100)				0.68
SNP442 G>A							
Group	**GG n (%)**	**GA n (%)**	**Total**		**95% CI**	**P value**	**HWE P value**
MUCOR	49 (100)	0 (0)	49 (100)		0.983-1.055	0.261	-
COVID	55 (98.2)	1 (1.8)	56 (100)				0.94
Total	104 (99.1)	1 (0.9)	105 (100)				0.96
SNP335 G>T							
Group	**GG n (%)**	**GT n (%)**	**Total**		**95% CI**	**P value**	**HWE P value**
MUCOR	49 (100)	0 (0)	49 (100)		0.993-1.125	0.05*	-
COVID	53 (94.6)	3 (5.4)	56 (100)				0.83
Total	102 (97.3)	3 (2.7)	105 (100)				0.88
****SNP 153C>T**							
Group	**CC n (%)**					**P value**	**HWE P value**
MUCOR	49 (100)					-	-
COVID	56 (100)					-	-

*P value is significant at P<0.05. All Ps were calculated by *t* test. ** Computed only for a 2×2 table; 95 % confidence intervals (95% CI), and P values for the association between SNPs and mucormycosis risk; HWE: Hardy-Weinberg

The Frequency of Alleles in COVID-19 Patients with and without Mucormycosis

The allele frequency of studied SNPs in COVID-19 patients with and without mucormycosis was also studied here (table 4). There were no significant differences in the allele frequency of SNP582 and SNP443 in COVID-19 patients with and without mucormycosis. The related statistical analyses for SNP442, SNP335, and SNP153 could not be performed due to the limited number of cases, and the analysis could only be computed for the 2×2 table.

The Association Between SNP582 A>G with the Risk of Mucormycosis and the Clinical and Laboratory Findings of COVID-19 Patients with Mucormycosis

Since SNP582 A>G is the well-studied

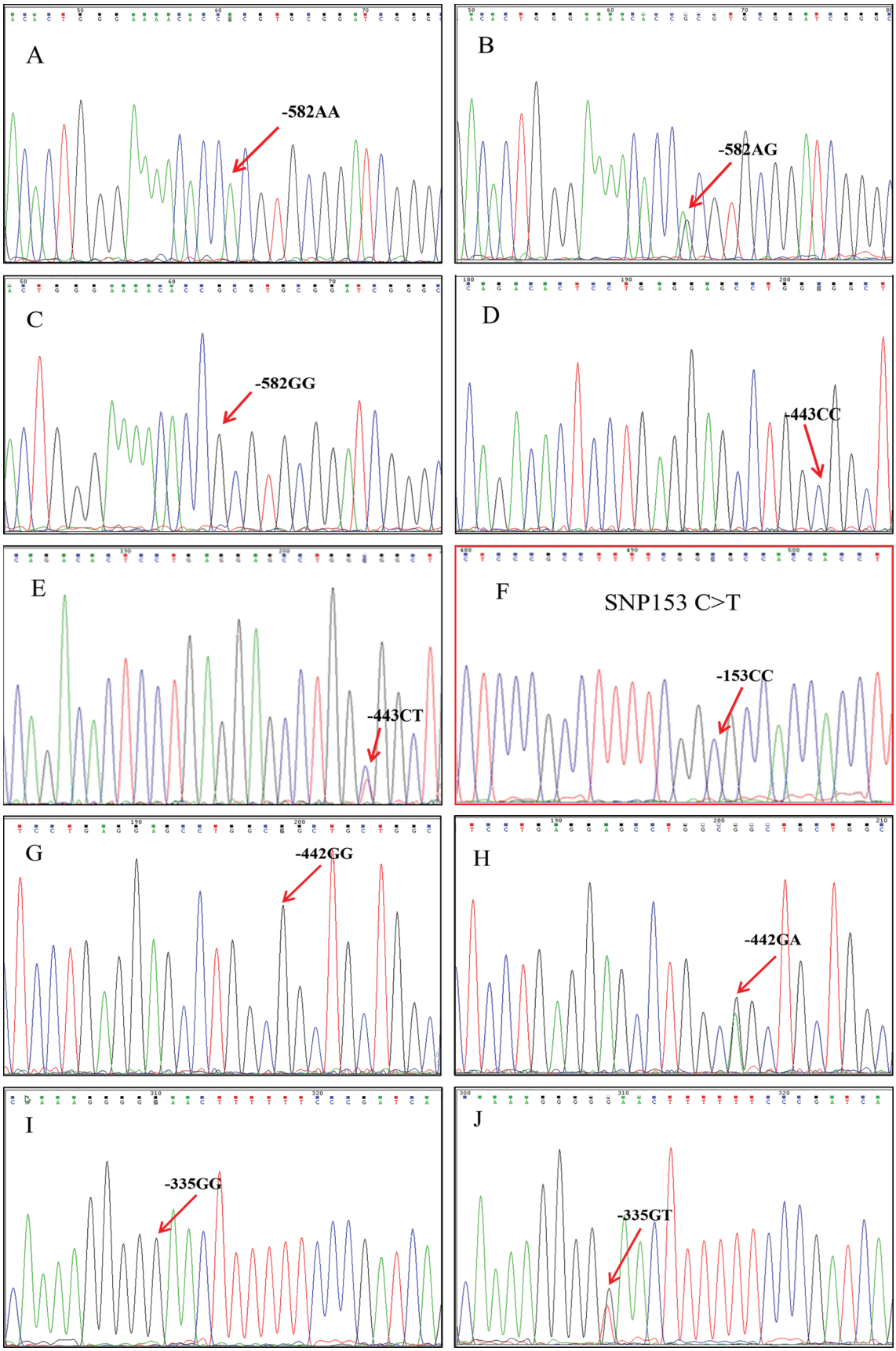

Figure 1: The sequencing diagrams of identified polymorphisms is shown in A to J. Diagrams A, B, and C show SNP582 C>G; diagrams D and E show SNP443 C>T; diagram F shows SNP153 C>T; diagrams G and H show SNP442 G>A, and diagrams I and J show SNP335 G>T. The red arrows indicate the detected polymorphisms. All diagrams were drawn by Chromas 2.6 software.

Table 4: Allele frequency in COVID-19 patients with and without mucormycosis

SNP 582 A>G					
Group	**A n (%)**	**G n (%)**	**OR**	**95% CI**	**P value**
MUCOR	75 (78.1)	21(21.9)	1.01	0.524 -1.968	0.97
COVID	87 (78.4)	24 (21.6)	1		
SNP 443 C>T					
Group	**C n (%)**	**T n (%)**	**OR**	**95% CI**	**P value**
MUCOR	92 (93.9)	6 (6.1)	3.43	0.707-18.2	0.1
COVID	110 (98.2)	2(1.8)	1		
SNP 442 G>A					
Group	**G n (%)**	**A n (%)**	**OR***	**95% CI**	**P value**
MUCOR	98 (100)	0 (0)	-	-	-
COVID	111 (99.1)	1 (0.9)			
SNP 335 G>T					
Group	**G n (%)**	**T n (%)**	**OR***	**95% CI**	**P value**
MUCOR	98 (100)	0 (0)	-	-	-
COVID	109 (97.3)	3 (2.7)			
SNP 153 C>T					
Group	**C n (%)**		**OR***	**95% CI**	**P value**
MUCOR	98 (100)		-	-	-
COVID	112 (100)				

*Computed only for a 2×2 table; Estimated relative risks with odds ratios (OR)

Table 5: Clinical and laboratory characteristics of patients and estimated P values for genotype–disease associations by clinical and laboratory status

Clinical and laboratory characteristics	**Groups**	**AA n (%)**	**AG+GG n (%)**	**Total**	**P value**	**Ref OR (95% CI)**
Age	<60	27 (49.1)	28 (50.9)	55	0.072	1
	≥60	32(66.7)	16 (33.3)	48		0.48 (0.22-1.07)
Sex	Female	38 (63.3)	22 (36.7)	60	0.139	1
	Male	22 (48.9)	23 (51.1)	45		1.8 (0.82-3.96)
Marital status	Single	6 (60)	4 (40)	10	0.792	1
	Married	35 (55.6)	28 (44.4)	63		1.2 (0.31-0.47)
PMH	No	9 (64.3)	5 (35.7)	14	0.842	1
	Yes	35 (61.4)	22 (38.6)	57		1.13 (0.34-3.82)
DM	No	19 (61.3)	12 (38.7)	31	0.983	1
	Yes	24(61.5)	15 (38.5)	39		0.99 (0.38-2.61)
Involved site	Rhinosinusitis	7 (70)	3 (30)	10	0.465	1
	Rhino-orbital-cerebral	6 (54.5)	5 (45.5)	11		1.94 (0.32-11.8)
WBC	Normal	49 (59)	34 (41)	83	0.446	1
	Leukocytosis	11 (50)	11 (50)	22		0.28 (0.11-1.2)
Hb	Normal	34 (63)	20 (37)	54	0.215	1
	Anemia	26 (51)	25 (49)	51		1.64 (0.75-3.56)
Plt	Normal	50 (58.1)	36 (49.9)	86	0.661	1
	Thrombocytopenia	10 (52.6)	9 (47.4)	19		1.25 (0.46-3.39)
Cr	Normal	40 (56.3)	31 (43.7)	71	0.888	1
	High	17 (54.8)	14 (45.2)	31		1.06 (0.46-2.48)
LDH	Normal	19 (51.4)	18 (48.6)	37	0.194	1
	High	22 (66.7)	11 (33.3)	33		0.53 (0.20-1.39)
LFT	Normal	29 (65.9)	15 (34.1)	44	0.95	1
	High	16 (66.7)	8 (33.3)	24		0.98 (0.34-2.77)
ESR	Normal	10 (62.5)	6 (37.5)	16	0.306	1
	High	14 (46.7)	16 (53.3)	30		1.91 (0.55-6.59)
CRP	Normal	7 (62.5)	8 (37.5)	15	0.328	1
	High	34 (46.7)	22 (53.3)	56		0.57 (0.18-1.78)

Estimated relative risks with odds ratios (OR) and 95 % confidence intervals (95 % CI) and P values for the association between SNP582 A>G and mucormycosis risk. The P value is significant at P<0.05. All Ps were calculated by *t* test. PMH: Past medical history, comorbidities other than DM including hypertension, hyperlipidemia, cerebrovascular accident, ischemic heart disease, chronic renal failure, hyperthyroidism, and cancer; DM: Diabetes mellitus; Ref: Reference line

pleomorphism of the hepcidin gene, the association between this polymorphism and the clinical and laboratory findings of COVID-19 patients with mucormycosis was investigated. There was a trend in which older patients were AA and younger patients were AG+GG in terms of SNP582 A>G. In our patients, there were no significant differences between the studied clinical and laboratory variables with this polymorphism (table 5).

Discussion

The results showed that increased levels of urea, AST, LDH, and PMN to lymphocytes were associated with a decreased risk of COVID-19-associated mucormycosis in COVID-19 patients. Furthermore, diabetes mellitus increased the risk of mucormycosis development in COVID-19 patients. It was found that none of the COVID-19 patients with mucormycosis displayed the SNP442 GA and SNP335 GT genotypes. However, some patients without mucormycosis demonstrated these genotypes. In terms of SNP 443 C>T, the CC genotype is associated with increased LDH levels in COVID-19 patients. Regarding SNP 582 A>G and SNP 443 C>T, COVID-19 patients with AA+CC genotypes had higher levels of LDH.

Mucormycosis is a rare, angio-invasive, opportunistic fungal infection, which has become a worrisome burden for COVID-19 patients following corticosteroid therapy. *R. oryzae* is the well-established causative fungus of mucormycosis in immunocompromised patients, including patients with diabetes mellitus. Hyperglycemia substantially impairs the function of immune cells, and low pH decreases the binding of iron to transferrin, shifting iron for fungal metabolism and the development of COVID-19-associated mucormycosis.[19] Iron is reported to be vital for the development of mucormycosis, and iron starvation activates programmed cell death in *R. oryzae.*[20] In line with this, it was reported that iron depletion can be an effective strategy for mucormycosis treatment.[21] Additionally, it was shown that iron chelation has a considerable protection effect against *R. oryzae* and improves the survival of diabetic ketoacidosis animal models.[22] Therefore, iron metabolism has a pivotal role in the development of COVID-19-associated mucormycosis. Regarding other clinical and laboratory factors, it was found that LDH, AST, and segmentation were positively associated with this condition, while increased levels of urea and lymphocytes were negatively associated with mucormycosis in patients with COVID-19. A positive association between ICU hospitalization and increased levels of LDH in COVID-19 patients with mucormycosis was shown.[23] Consistent with previous findings,[24] diabetes mellitus significantly increased the risk of mucormycosis development in our COVID-19 patients. Our results indicated that elevated levels of urea, AST, LDH, and increased ratio of PMN to lymphocytes were associated with decreased risk of COVID-19-associated mucormycosis in COVID-19 patients.

Hepcidin is a major regulator of iron homeostasis, which is produced in hepatocytes. Hepcidin expression is regulated according to the level of iron in a negative feedback loop manner.[25] In infections and inflammations, hepcidin expression is increased to modulate inflammation and decrease the available iron for pathogens. In COVID-19 patients and patients with septic shock, the serum level of hepcidin is substantially elevated compared to healthy individuals.[26] A recent meta-analysis study indicated that severe COVID-19 patients have higher hepcidin levels than those with non-severe conditions.[27] Aside from regulating iron homeostasis, hepcidin increases inflammation *via* the IL-6 effect.[10] Moreover, *Rhizopus arrhizus* var. delemar can express peptides that regulate hepcidin expression in blood-derived monocytic macrophages.[12] In addition to the association of hepcidin with inflammation, increased levels of hepcidin decrease iron availability for mucormycosis development. Therefore, the role of hepcidin gene polymorphisms in the context of COVID-19-associated mucormycosis was investigated in this study.

Polymorphisms are genetic variations that can interact with epigenetic factors, such as DNA methylations, microRNAs, and transcription factors, and modulate gene expression.[28]

The SNP 443 C>T, SNP 442 G>A, SNP 335 G>T, and SNP 153 C>T are the polymorphisms located in the promotor of the *HAMP* gene. SNP582 A>G, one of the well-studied polymorphisms of *HAMP*, in the presence of G allele decreases hepcidin expression.[16] Regarding the SNP582 A>G, Liang and colleagues found that $CD14^+$ monocytes with the GG genotype had decreased hepcidin expression compared to cells with at least one A allele.[15] Our results showed that none of the COVID-19 patients with mucormycosis display the SNP442 GA and SNP335 GT genotypes. However, some patients without mucormycosis demonstrate these genotypes. In terms of clinical and laboratory findings, SNP443 C>T (rs117345431) was associated with LDH levels in COVID-19 patients, and the CC genotype was significantly associated with higher levels of LDH in the affected patients (figure 2).

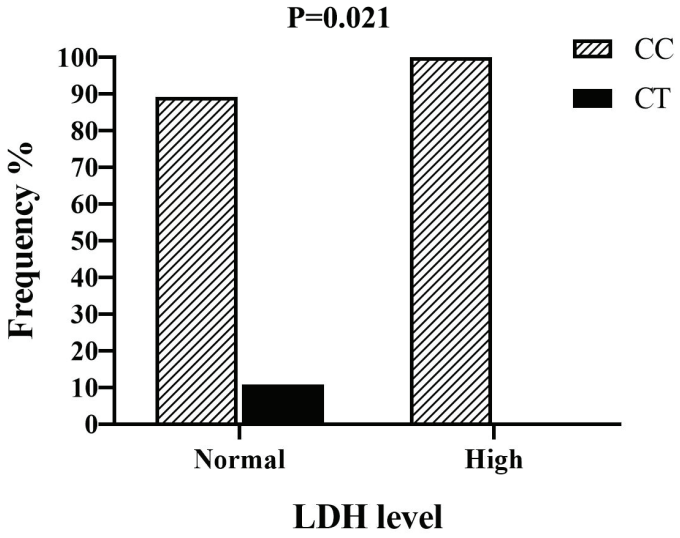

Figure 2: Association of SNP 443 C>T with LDH levels. As indicated in the chart, the CC genotype was significantly associated with higher levels of LDH in the affected patients.

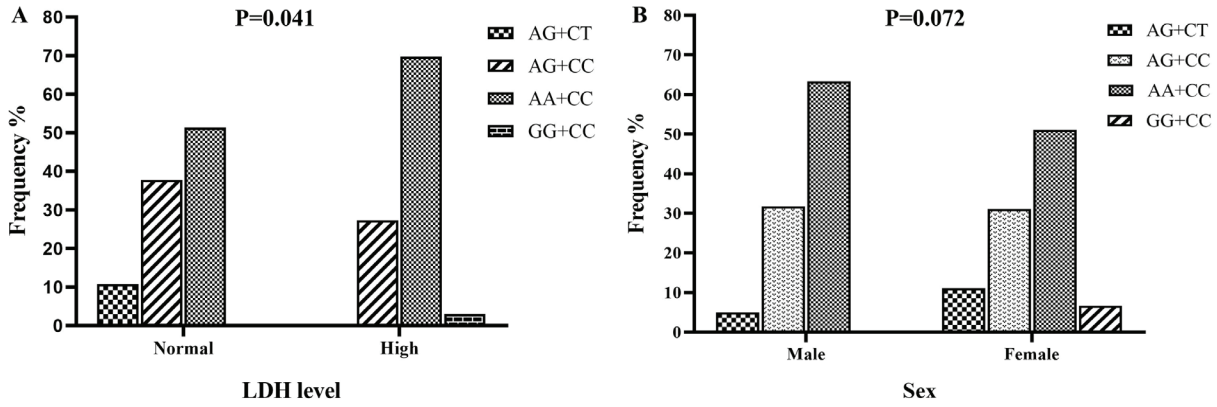

Figure 3: Association of the combination effect of SNP582 A>G and SNP 443 C>T on LDH levels and sex. Figure 3A shows that COVID-19 patients with AA+CC genotypes had higher levels of LDH, and the patients with AG+CT genotypes had normal levels of LDH. Figure 3B depicts that there is a trend toward significance for males with AA+CC genotypes.

Regarding the rs10421768 and rs117345431 polymorphisms, COVID-19 patients with AA+CC genotypes have higher levels of LDH and patients with AG+CT genotypes have normal levels of LDH (figure 3A). Moreover, it is worth mentioning that there was a trend toward significance for sex (figure 3B).

The current study has several strengths. Given the significance of hepcidin and iron metabolism in infection, inflammation, and COVID-19-associated mucormycosis development, it is the first study to investigate the significance of hepcidin polymorphisms in COVID-19-associated mucormycosis. Second, we thoroughly investigated included patients' clinical and laboratory features and medical history to study the potential risk factors in COVID-19-associated mucormycosis. However, the current study has several limitations as well. First, this study is retrospective. Moreover, we were unable to investigate the prognostic values of hepcidin polymorphism in affected patients.

Overall, the current study provides valuable insights into the role of hepcidin polymorphisms in developing COVID-19-associated mucormycosis.

Conclusion

Unlike COVID-19 patients without mucormycosis, none of the COVID-19 patients with mucormycosis had the SNP442 GA and SNP335 GT genotypes. Regarding SNP 443 C>T, the CC genotype was associated with increased LDH levels in COVID-19 patients. In terms of SNP 582 A>G and SNP 443 C>T, COVID-19 patients with AA+CC genotypes had higher levels of LDH. Regarding the clinical and laboratory results, increased levels of urea, AST, LDH, and PMN to lymphocytes were associated with a decreased risk of COVID-19-associated mucormycosis in COVID-19 patients. Besides, diabetes mellitus increased the risk of mucormycosis development in COVID-19 patients.

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Authors' Contribution

R.R, Y.F, F.R.G: conception and design of the work; Y.F, F.R.G: the acquisition of Data; R.R, F.R.G: analysis and interpretation of data; Y.F, F.R.G: drafting the work; R.R, F.R.G: reviewing it critically for important intellectual content. All authors have read and approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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