Matrix Metalloproteinase-3 and Tissue inhibitor of metalloproteinase-2 as Diagnostic Markers for COVID-19 Infection

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Abstract

   Coronavirus 2 is the cause of coronavirus disease 2019 (COVID-19), which leads to severe acute respiratory illness. Matrix metalloproteinases (MMPs) have been linked to leukocyte infiltration and chemokine activation during inflammatory responses. Tissue inhibitors of metalloproteinase (TIMP) family are thought to dampen the proinflammatory effects of these MMPs. The molecular pathways of lung fibrosis are mediated by MMPs and TIMPs. In this study, we sought to investigate the probable link between MMPs, specifically MMP-3, TIMP-2, and COVID-19. The study included 58 COVID-19 patients and 30 apparently healthy individuals matched in terms of age and sex. Multiplex real- time PCR was used to detect the ORF1ab, E, and N genes of SARS-Cov-2, ELISA was used to evaluate the quantities of soluble MMP-3, TIMP-2, and C reactive protein in serum. The results showed that the serum levels of MMP-3 and TIMP-2 were noticeably higher in COVID-19 patients than healthy controls; which and was statistically significant (p <0.001). Estimation of serum MMP-3 and the inhibitor TIMP-2 may have a useful indication for COVID-19 diagnosis.

Keywords: COVID-19, MMP-3, TIMP-2, diagnosis, ELISA

انزيم البروتين المعدني –3 والمثبط النسيجي للبروتين المعدني–2 كمؤشرات تشخيصية للإصابة
بكوفيد –19

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الخلاصة

يعد فيروس كورونا 2 السبب لمرض كورونا 2019(كوفيد–19)والذي يسبب مرضا تنفسيا حادا , وجدت الدراسات ان هناك علاقة بين البروتينات المعدنية المصفوفة ودورها في ارتشاح الكريات البيض وتفعيل الكيموكين اثناء الاستجابات الالتهابية، يعتقد ان مثبطات الانسجة لعائلة البروتينات المعدنية تعمل على تثبيط التاثيرات الإلتهابية لهذه البروتينات و ان المسارات الجزيئية التي تقود الى تليف الرئة تتوسطها البروتينات

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المعدنية ومثبطات الانسجة . عمدت هذه الدراسة للتحري عن وجود صلة محتملة بين البروتينات المعدنية وتحديدا (3) ومثبطات الانسجة (2) والاصابة بفايرس كوفيد –19. تضمنت هذه الدراسة 58 مريضا مصاب بفايرس كوفيد –19 من الراقدين في المستشفى وثلاثين شخصا من الاصحاء ظاهريا، تم استخدام تفاعل البلمرة المتسلسل للكشف عن جينات المغلف والنيكليوكابسد واطار القراءة المفتوح لفايروس (سارس-كوف-2) , كما تم استخدام تقنية الامتزاز المناعي المرتبط بالانزيم لتقدير مستويات البروتينات المعدنية3 ومثبط الانسجة 2 وكذلك بروتين الطور التفاعلي C في المصل. اظهرت النتائج ان المستويات المصلية لبروتينات المعدنية 3 ومثبطات الانسجة (2) كانت اعلى بشكل ملحوظ في مرضى كوفيد-19 مقارنة بالاشخاص الاصحاء مع وجود فروق ذات دلالة احصائية عالية . نستنتج من هذه الدراسة انه قد يكون تقدير مصل البروتينات المعدنية 3 ومثبط الانسجة 2 ذو دلالة مفيدة في تشخيص كوفيد-19.

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Introduction

A recently Discovered respiratory sickness, coronavirus disease 2019(COVID-19) which caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has fast spread all over the world, as it was   declared by World Health Organization (WHO) as pandemic in March 2020 [1]. Until now, the viral nucleic acid test has been considered as the most used approach for detecting COVID19. A structural protein of SARS (S) contains spike protein, membrane glycoprotein (M), nucleocapsid protein (N) and envelope protein (E). M glycoprotein is the most prevalent structural protein in coronaviruses, and it can bind to any structural protein. Binding with M protein aids in the stability of N proteins and enhances viral assembly finalization by fixing the N protein-RNA complex within the virion[2].

The host angiotensin-converting enzyme 2 (ACE2) is used as an entrance receptor by the SARS-CoV-2 spike protein [3]. Mutations in M proteins which works with S proteins may alter the virus adhesion and entrance into the host cell. Growing evidence have suggested that endo-proteinases (particularly matrix metallo-proteinases (MMPs) regulate both processes [4]. The MMPs are a family of 23 secreted endo-proteinases that digest extracellular matrix contains (collagens, fibronectin, and elastin), growth factors, cytokines, and chemokines, [5]. Tissue inhibitors of metallo-proteinases (TIMPs) can impede their activity selectively, where the balance of MMPs/TIMPs is important for maintaining normal tissue structure and the physiological functions [6].

The MMP levels in normal adult tissues are normally low, and their secretion and efficacy are controlled to substantially unnoticeable levels with a few exceptions. MMPs have long been suspected of playing a role in pulmonary fibrosis development, causing a structural damage and airway remodeling [7]. The MMPs are mostly expressed in sick, inflamed, or undergoing repair and remodeling tissues, rather than in normal healthy tissues. Because pulmonary epithelial cells produce numerous forms of MMPs, they could be a large source of MMPs [8]. The MMP3 (stromelysin-1) is a zinc-dependent endopeptidase that belongs to a large family of MMPs. A recent study has found that MMP3 could affect respiratory disorders like lung cancer, pulmonary fibrosis, acute respiratory distress syndrome (ARDS), and acute lung injury (ALI) [9].

TIMPs are a group of proteins that suppress matrix MMPs and regulate MMP-mediated activities such cytokine processing, growth factor binding protein degradation, and ECM-bound growth factor synthesis [10]. The TIMP-1, -2, -3 and -4 are members of the TIMP family which have structural similarities and the potential to inhibit MMPs [8]. The TIMP-1 and -2 are soluble proteins, while TIMP-3 is associted with the extracellular matrix [11].

Based on these characteristics and their crucial role in acute lung injury, MMPs have recently been postulated as a feasible diagnostic/therapeutic in COVID-19[12]. However, few studies have been conducted regarding the link between MMP-3, TIMP-2 and COVID-19. The current study assessedserum levels of MMP-3 and TIMP-2 to determine if these inflammatory mediators have a remarkable role in COVID-19 diagnosis.

Subjects and methods:
COVID-19 infected patients participated from several hospitals in Baghdad according to the protocol granted by the Ethical Committee, (University of Baghdad- College of science- department of Biology Bioethics Committee Ref. no. CSEC/1121/0065.

According to the nasopharyngeal swab positive results, fifty-eight patients were enrolled, as well as thirty healthy individuals matched in terms of age and sex; with a negative nasopharyngeal swab. Healthy controls were excluded if they had a history of systemic autoimmune illness, previous infection with SARS-CoV-2, had allergy diseases, or were pregnant.

Molecular detection of SARS-CoV-2 by multiplex PCR
SARS-CoV-2 is detected molecularly using a multiplex real-time reverse transcription PCR technique with particular primers and fluorescent probes that target the ORF1ab, E, and N genes of the virus. In brief, virus nucleic acid is detected by monitoring fluorescence intensity in real time, internal control is added to monitor the presence of PCR inhibitors within a specimen, thus effectively preventing false negative results. Maccura Biotechnology Co.LTD. Chengdu,China).

Specimen collection and preparation
• The oropharyngeal swabs were collected and placed in a disposable viral sampling tube with virus preservation solution, with the tail and tightened cover discarded.

• The specimen tested as soon as feasible after being stored. Specimens can be stored for 24 hours at 2°C-8°C or for 3 months at -70°C, with a maximum of five freeze-thaw cycles.

• Virus inactivation: For inactivating the virus, the constant temperature water bath equipment was heated to 56°C in advance, then the sealed package containing the specimen was sprayed with 75% ethanol in a biosafety cabinet class II, the specimen was placed at room temperature for 30 min. The collection tube was prevented from floating, and the specimen was gently blended for 10 min.

Assay procedure
• All kits reagents components including nucleic acid extraction or purification kit were brought to room temperature prior to use and mixed gently.

• qRT-PCR mix was prepared according to the manufacture instructions of Maccura biotechnology Co. LTD. Chengdu, China.

• The volume used to conduct the reaction of qRT-PCR for gene amplification was totally 20 µL where (17 µL of qRT-PCR reaction mix + 3 µL of qRT-PCR enzyme mix)

• Nucleic acid extraction.
Two µL of internal control per test were transferred into a negative control, positive control, and specimen for nucleic acid extraction.

The recommended volume of extraction and elution were (200 µL + 35 µL) according to Maccura Mag-Bind RNA extraction kit and (140 µL + 80 µL) according to QIA amp viral Mini kit respectively.

• Specimens addition
Twenty µl of RNA templates (nucleic acid extracted from the negative control, positive control, and specimen) were added to a PCR reaction tube containing qRT-PCR mix, with a total volume of 40 µl test. To avoid contamination, the plate lid was promptly secured.

• PCR amplification
• Reaction volume : 40 µL

• The cycling protocol was set as the following: 1 cycle for reverse transcription at 55°C for 15 min, 1 cycle for Tag polymerase activation, pre-denaturing at 95°C for 2 min, 40 cycles for denaturation at 95°C for 15 sec, 40 cycles for annealing, extension, florescence acquisition at 58°C for 35 sec, and 1 cycle for instrument cooling at 40°C for 10 sec.

The results were saved when the run was completed. Threshold level, start value and end value of baseline of a different channel could be manually adjusted as follows: start value sets to 3-15, end value sets to 5-20. Threshold level should be adjusted according to fluorescence background and negative control (NC) higher than fluorescence background and NG, FAM, ROX, CY5 amplification curve of NC should be horizontal or lower than a threshold level. "analyze" was clicked and the results were presented on the report screen.

Detection of MMP-3and TIMP-2 by ELISA procedure

Soluble MMP-3 (Catalogue No. SL 1152Hu), TIMP-2 (Catalogue No. SL 1712Hu), and CRP (Catalogue No. SL0535Hu) levels in serum were measured using quantitative human ELISA Kit as described by the manufacturing company (SunLong Biotech Co., LTD).

Statistical analysis

The Statistical Analysis System- SAS version 24 program was used to analyze the study data. The used tests were ANOVA, T test as well as Person test. $P<0.05$ was considered statistically significant [13].

Results

This study comprised a total of 58 cases of COVID-19 and 30 healthy participants.

Their ages mean was 58.59 ± 15.58 and 56.41 ± 14.72 years respectively.

Detection of SARS-Cov-2 infection by an envelope (E) gene and nucleocapsid (N) gene

The molecular diagnosis was confirmed using RT-PCR; although the kit uses a multiplex PCR technique, gene up-stream and down-stream ends of particular primers were incorporated when using envelope (E) and Nucleocapsid (N) genes specific primers. All the patients included in the study were positive for real-time PCR.

MMP-3, TIMP-2 and C-reactive protein detection by ELISA

MMP-3 serum levels in the COVID-19 patients group and control group are shown in Figure 1. The mean concentration of MMP-3 was 329.5 ± 106.48 pg/mL in the patients group and 227.83 ± 73.14 pg/mL in comparison to the control group and there was a vast variation between these two groups ($p<0.001$); while TIMP-2 concentration mean was 452.4 ± 167.95 pg/mL and 192.59 ± 46.5 pg/mL in the patients and control group respectively, and the differences were also statistically highly significant between the two groups ($p<0.001$) as shown in Figure 2.
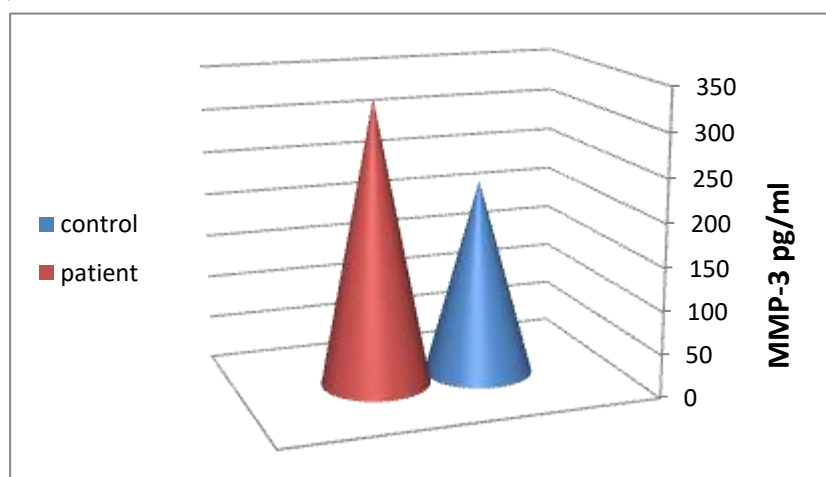

Figure 1-The mean level of MMP-3 (pg\ml) in sera of COVID-19 patients and control groups.
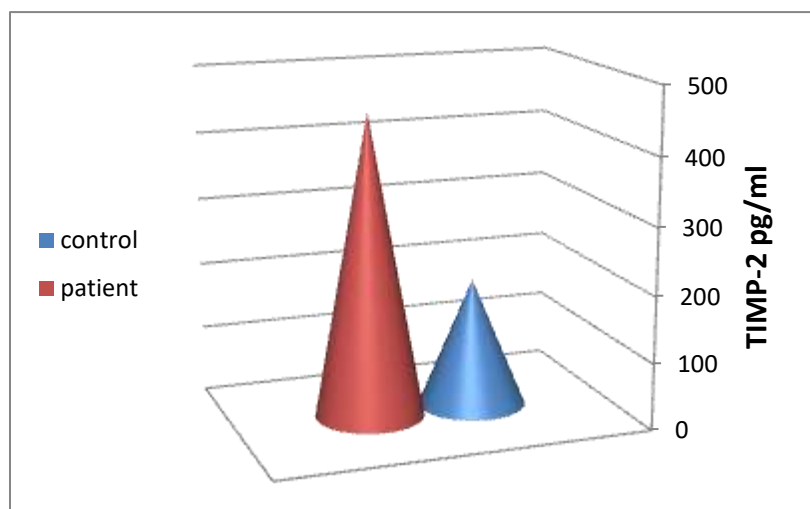
Figure 2 - The mean level of TIMP-2 (pg\ml) in sera patients infected with COVID-19 and control groups

The mean level of CRP was statistically significant in COVID-19 patients, it was 45.12±10.11 pg/ ml in the patients group versus 2.5±0.59 pg/ ml in the control group (p<0.001).
Table 1 shows the patients test findings. COVID-19 patients had a substantially higher total leukocytic count in their blood (12.11±5.14) than the control group (5.75±1.67). The patients' erythrocyte sedimentation rate (ESR) and random blood sugar (RBS) were statistically significantly higher than the controls'. However, no significant differences in haemoglobin (Hb) level, platelets number, or body mass index (BMI) were found between the two groups.

Table 1- Laboratory results of COVID-19 patients and healthy groups.

Parameter (unit)	Control (N=30)	COVID-19patients (N=58)	p-Value
WBC (cmm)	5.75±1.67	12.11±5.14	<0.001**
Hb (g/dl)	15.53±2.44	15.21±12.9	0.89 NS
Platelets (cmm)	286.30±102.23	282.19±136.67	0.89 NS
ESR (mm/hr)	10.4±6.1	59.02±27.99	<0.001**
RBS (mg/dl)	94.7±12.85	226.2±116.58	<0.001**
BMI (kg/m^2)	28.51±12.38	28.03±9.24	0.71 NS

** Highly significant=less than 0.01, NS: Non significant

Discussion
The COVID-19 pandemic has added further momentum to the annual mortality toll from illnesses that are infectious since few years, posing a remarkable menace to human health around the world [14]. The corona viral genome encodes the spike (S) protein, nucleocapsid (N) protein, membrane (M) protein, and envelope (E) protein, all of which are necessary to generate a structurally entire viral particle as SARS-CoV-2 molecular diagnostics [15].
The E protein is the tiniest and most enigmatic of the main structural proteins. The protein is mostly located in intracellular locations such as the endoplasmic reticulum and Golgi apparatus which helps in CoV replication [16]. Recombinant CoVs missing E protein

revealed much lower viral titers, are unable to mature and create reproduction-ineffective progeny, emphasizing the significance of E in virus growth and ripeness [17]. The (N) gene is more stable, with 90% homology of amino acid and fewer changes over the season; numerous coronavirus (N) proteins are extremely immunogenic and are produced in large amounts after hitting. The (N) protein is an ideal antigen for the cellular response in a vaccination tune, promoting virus-specific T-cell propagation and cytotoxic action [18]. By encouraging SARS-specific T-cell propagation and cytotoxic action, the N protein appears as a typical antigen for the T-cell response [19]. Although the genome has been adequately articulated, plenty is still unknown. Comparing results from the current investigation to a prior studies on the prediction effect of MMPs and TIMPs in infections or critically ill patients is difficult since there are differences in patient details, illness classification, exclusion criteria, (MMP) or (TIMP) [20]. Moreover, as compared to earlier findings, the current study is one of the few researches that investigated the MMP-3 and the first to highlight the TIMP-2 and its correlation with covid-19. Hoffmann *et al.*[21] discovered that the novel coronavirus (SARSCoV2) uses ACE2 and serine protease to enter host cells, similar to SARSCoV. Because the virus infects cells through a complex process involving some proteases, it is crucial to look into antiviral intervention through correlative proteases. A recent study by Phillips *et al.* [22], focused on the role of numerous protein degrading enzymes in infection with coronavirus, where it was indicated that zinc-dependent metalloproteases, such as MMP, could play a role in coronavirus fusion. As a result, it is reasonable to assume that MMP-3 is involved in SARSCoV2 infection of host cells through cell incorporation. TIMPs are thought to achieve a key role in redesigning after parenchymal injury, which leads to tissue loss or the activation of healing mechanisms in pulmonary disease [8]. Shengjie Shi *et al* [9] recently published a study that found higher MMP-3 levels in COVID-19. MMP-3 is mostly produced by endothelial cells and fibroblasts, although it can also be triggered by inflammatory cells and cytokines. The MMP-3 non-matrix substrates are pro-inflammatory cytokines like interleukin 1 (IL-1) and tumor necrosis factor (TNF), as seen in pulmonary infection with *Mycobacteria* and *Chlamydia* which promote the production of a variety of cytokine and chemokine signals by host immune cells, thus achieve critical strategies against infection with Mtb [23-26].

In addition, MMP-3 activates additional MMPs such as pro MMP1, 3, 7, 8, 9, and 13. While MMP3s proteolysis role contributes to intracellular migration by the MMP3 which is not only rubbed adhesion points between cells and the matrix, but it also regulates the action of cytokines and chemokines, affecting disease progression to a certain degree [9].

Another study found that infection with mouse hepatitis virus (MHV), where overexpression of MMP-3, MMP-8, and MMP-14 mRNAs was noticed in the same family of beta-coronavirus as SARS-CoV, MERS-CoV, and SARS-CoV-2 [27].

Leukocytes and endothelial cells produce MMP-3 (stromelysin), which degrades a set of collagen substances. MMP-3 has been linked to asthma, acute respiratory distress syndrome (ARDS) and idiopathic pulmonary fibrosis. Inhibition of MMP3 has been demonstrated to dampen the severity of LPS-induced ARDS in rats, as well as the idiopathic pulmonary fibrosis [4, 28]. A study on mice reported that TIMP-1 main expression is low but rapidly elevated in response to a variety of inflammatory signals; TIMP-2, -3, and -4, on the other hand, are constitutively expressed, and variations in their expression are slightly linked to inflammatory reactions [27]. The mRNA expression of MMP-3, MMP-12 and TIMP-1 has been demonstrated to correlate with viral pathogenicity and viral load in earlier studies [29]. During demyelinating illness, expression of T1MP-1 has been linked to tissue inhabitant cells and the majority of inflammatory cells [20]. Our research can contributes to a better understanding of TIMP-2s function. According to the current investigation, a high level of TIMP-2 may be an aspect of a conventional host defense strategy against overexpression of several metalloproteases induced by the virus [30].

This could be explained in part by the presence of additional proteases as well as the huge number of MMPs with overlapping characteristics. Consequently, whereas MMP expression is linked to inflammatory responses in demyelinating illness, the role of particular MMPs as active participants or just as indicators is unknown [27]. We found a significant rise in both MMP-3 and TIMP-2, which is consistent with prior studies. A recent Current literature on COVID19 emphasized the involvement of inflammation and immunological responses in COVID19, and showed that MMP-3 and inflammatory mediators like (IL-1) and (IL-6) have a positive relationship. The immunological response of the host to the SARS CoV2 infection from the perspective of protease and immunological protection is remarkable [9].

A higher prevalence of acute kidney damage (AKI) and high TIMP-2 level were seen in COVID-19 patients who required intensive care, the worst outcomes among critically sick patiens [31]. Alterations in the levels of several blood indicators have been associated with the severity and death of COVID19 patients in clinical investigations. In COVID19 individuals with severe symptoms, serum C reactive protein (CRP) has been considered a critical measure that increased [32, 33].

On the other hand, other research revealed an increase in white blood cell count [31]. Patients with greater WBC levels were found to have a higher risk of death in a recent study [34]. Furthermore, the SARS coronavirus has been shown to infiltrate pancreatic islets and kill them, resulting in acute hyperglycemia [2], as our findings evidenced. Ultimately, serum MMP-3 and TIMP-2 levels can possibly be considered as valuable diagnostic tools for COVID-19 patients. This study finding could also aid in the monitoring, diagnosis, and potential therapy of COVID19.

Conclusion

The results showed that the detection of MMP3 and TIMP-2 may be the similar to other investigated inflammatory factors, which may focus on the inflammatory process as a part of SARS-CoV-2 infection events. So, the detection of serum MMP-3 and TIMP-2 levels may be helpful tools in the identification of COVID-19 patients. The results obtained from the current study may also be valuable in COVID-19 follow up as well as therapeutic purposes.

Conflict of interest

The authors have no conflicts of interest to declare.

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