Correlation between interleukin-6 expression

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Original Article

Correlation between interleukin-6 expression in post-mortem core liver biopsy and degree of liver injury in patients with fatal COVID-19

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Abstract

Excessive release of interleukin-6 (IL-6) during the progression of coronavirus disease 2019 (COVID-19) induces cytokine storms, resulting in multi-organ damages including liver injury, similar in nature with mechanism of viral hepatitis. Systemic IL-6 has been associated with the incidence of liver injury among COVID-19 patients; however, studies on IL-6 expression in the liver tissue are completely lacking. The aim of this study was to measure the IL-6 expression in the liver tissues and to determine its correlation with the degree of liver injury in fatal COVID-19 patients. Through this first cross-sectional study, IL-6 expression was measured through immunohistochemical staining and the degree of liver injury was identified based on level of serum alanine aminotransferase (ALT). The Spearman correlation test was used to identify the correlation between IL-6 expression and the degree of liver injury. A total of 47 deceased COVID-19 patients were included and IL-6 expression was observed in all post-mortem liver specimens, ranging from mild to strong expression. Liver injury at various degrees (mild to severe) was found in more than half (59.5%) of the cases. The Spearman correlation analysis suggested a statistically insignificant correlation between liver IL-6 expression and the degree of liver injury (r=0.152; p=0.309). In conclusion, even IL-6 expression was observed in all post-mortem liver specimens, there was an insignificant correlation between IL-6 expression in the liver tissue with the degree of liver injury among fatal COVID-19 patients, suggesting that IL-6 was not the only main factor contributing to liver damage in COVID-19 patients.

Keywords: Core biopsy, interleukin-6, liver injury, post-mortem, hepatitis

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Introduction

Multiple organ damages among coronavirus disease 2019 (COVID-19) patients have been widely reported, including liver injury [1-3]. Approximately 46%–62% of patients with critical to fatal COVID-19 suffer from liver injury [4] and 14%–54% of severely ill COVID-19 patients experienced increased levels of serum alanine aminotransferase (ALT) which indicates a process of inflammation or damages in hepatocytes as a result of interaction between Kupffer cells and cytotoxic T-cell in which causing another condition that is similar with viral hepatitis [5-8]. This complication contributes to prolonged hospital stays and results in a three-time higher incidence of mortality among COVID-19 patients compared to those without liver injury [9-12].

Many factors have been thought to contribute to liver damage in COVID-19 patients, including the cytopathic effect of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19, on hepatocytes and cholangiocytes, drug consumption during therapies, a history of comorbidities, and hyperinflammatory events associated with cytokine storms [13-15]. Uncontrolled inflammation due to the presence of cytokine storms has been associated with the deterioration of COVID-19 conditions in infected individuals [1]. In patients with severe to fatal COVID-19, cytokine storms may lead to various complications, such as alteration of permeability, vascular diseases, hypercoagulopathy, multi-organ damage such as liver injury [13-15] and even contribute to an increased rate of mortality [4,16]. Cytokine storms occur as a result of excessive release of various inflammatory factors [9,17].

Interleukin-6 (IL-6) is a hallmark cytokine of fatal COVID-19 and plays a role in the pathogenesis of COVID-19 [9,17-19]. Approximately 80% of patients with severely to critically ill COVID-19 have been reported to experience cytokine storms related to a rise in IL-6 [14]. IL-6 is not only important in the induction of acute response in the liver but also plays a role in hepatocyte homeostasis [9,20]. However, whether increased IL-6 expression in the liver during cytokine storms contributes to liver injury in COVID-19 patients remains unclear. Therefore, the aim of this study was to identify the correlation between IL-6 expression in the liver tissue with the degree of liver injury in patients with fatal COVID-19 through post-mortem core biopsy.

Methods

Study design and population

A cross-sectional study was conducted among fatal COVID-19 patients hospitalized during the period of July-December 2020 at Dr. Soetomo General Academic Hospital, Surabaya, Indonesia. COVID-19 patients ≥18 years of age passing away due to COVID-19 during the period of treatment, and had their core liver biopsy paraffin blocks preserved at Anatomical Pathology Laboratory of Dr. Soetomo General Academic Hospital were included in the study. Written informed consent was obtained from family members of each patient.

Study variables

This study evaluated the expression of IL-6 in the liver tissue as the independent variable and the degree of liver injury based on the level of ALT was the dependent variable. IL-6 expression was defined as the percentage of hepatocytes stained with polyclonal IL-6 antibody (GTX17623). IL-6 expression was evaluated semi-quantitatively using a 4-level point system based on the number of stained hepatocytes (0 = no expression; 1 = positive expression in <30%; 2 = positive expression in 30%–70%; and 3 = positive expression in ≥70% of cells) [21]. IL-6 expression was determined by two experienced anatomical pathologists at the Anatomical Pathology Unit of Dr. Soetomo General Academic Hospital, Surabaya, Indonesia.

The degree of liver injury in COVID-19 was determined based on the extent of serum ALT exceeding the upper limit of the normal range [22] and was classified into five categories: no liver injury (no increase in ALT level); mild (ALT level of <2 times the normal limit); moderate (ALT level of ≥2 to <5 times the normal limit), and severe (ALT levels of ≥5 times the normal limit). Data on patients' serum ALT were collected during the patients' terminal phase and were approved by a clinical pathologist. The levels of ALT were measured using a Siemens Dimension® EXL™ 200 Integrated Chemistry System machine using the IFCC (PSP) method, with the normal ranges of 0–50 u/L for men and 0–35 u/L for women.

In addition, some covariates were also collected. Data on patients' demographics and clinical profiles were collected during the treatment course. It included age, sex, duration of treatments, hematologic parameters related to inflammation and COVID-19 severity (serum leukocyte, neutrophil, lymphocyte, neutrophil-to-lymphocyte ratio (NLR), ferritin, D-dimer, C-reactive protein (CRP), and procalcitonin), several indicators for liver injury (serum aspartate aminotransferase (AST) and albumin), drugs given for COVID-19 treatments, comorbidities, and causes of death.

Immunohistochemical staining of IL-6 expression

A liver biopsy was done in post-mortem in all cases. The patients' core biopsy of liver tissues then preserved at the Anatomical Pathology Laboratory at Dr. Soetomo General Academic Hospital, Surabaya, Indonesia. About 3–4 µm incision of paraffin tissues were histopathologically proceeded and underwent IL-6 polyclonal antibody staining. Immunohistochemical staining was performed using the labelled streptavidin biotin II procedure, with neutral buffer formalin 10% as a fixative agent. IL-6 expression was measured using IL-6 antibody (GTX17623, Genetex, CA, USA). In brief, the immunohistochemistry (IHC) slide was deparaffinized in three different xylol solutions for five minutes each. The slide was then transferred through 96% and 80%, respectively for two minutes each before being rinsed thoroughly using distilled water for five minutes. A proxy block was added onto the slide and let stand for 15 minutes to block endogenous peroxidation. The sample was then rinsed with distilled water for five minutes. Antigen retrieval was performed by heating the slide at 950°C for 20 minutes in a decloaking chamber supplemented previously with buffer citrate (pH 6). After the buffer cooled down at room temperature for five minutes, the slide was rinsed respectively with distilled water and phosphate buffer citrate (PBS) for five minutes each.

Main staining was performed using IL-6 antibody in a 1:100 ratio (overnight incubation in a magnetic immunostaining at 40°C), followed by trekke universal link, trekavidin-HRP label, and 3,3′-diaminobenzidine. Counterstaining was carried out by incubating the slide in Meyer's hematoxyllin for five minutes at room temperature. After being washed, the slides were dehydrated in alcohol solution with gradual increase in concentrations and immersed in three Xylol solutions and mounted using EZ-mount.

IL-6 expression was determined by observing the color of antibody staining in the tissue sections under a light microscope (Olympus CX22LED) with 100x and 400x magnifications. The results were assessed and confirmed by two anatomical pathologists using a 4-level point system based on the number of stained hepatocytes as explained.

Statistical analysis

The numerical variables, demographic characteristics, and clinical findings were provided as percentage (%), mean±standard deviation (SD), and median (min-max). The inter-rater reliability for histochemical staining results was measured using the inter-observer agreement using the Kappa statistical method. The Spearman correlation test was used to identify the association between IL-6 expression in liver tissue and the degree of liver injury in fatal COVID-19 patients. A p-value of <0.05 was considered statistically significant. SPSS software for Windows version 25.0 was used to analyze the data (IBM, Armok, New York, USA).

Results

Patients' characteristics

A total of 47 critically ill COVID-19 were enrolled and analyzed in the study. The patient's demographic and clinical profiles related to COVID-19 and liver function are summarized in **Table 1**. The mean age of the patients was 48.8 years and the majority (76.6%) of the patients were males. The average duration of hospitalization was 10.96 days. Hematological analysis revealed increased levels of leukocyte, neutrophil, lymphocyte, and NLR. Elevated AST (mean 218.8 u/L), ALT (mean 198.4 u/L), and decreased of albumin (mean 2.7 g/dL) levels suggested liver dysfunction. The levels of the inflammatory markers such as ferritin, D-dimer, procalcitonin, and CRP were also elevated (**Table 1**).

Interleukin-6 expression

The mean score of IL-6 expression in patients' liver tissue was 62.49% (min-max: 25%–90%), suggesting moderate expression in general. The expression of IL-6 in the patients' hepatocytes of each grade is presented in **Figure 1**. The distribution of IL-6 expression in liver tissues and the degree of their liver injury are presented in **Table 2**. IL-6 expression was positive in the liver tissue of all patients. More than half (57.4%) of the liver specimens had moderate IL-6 expression (30%–70%), whereas 40.4% exhibited strong expression (≥70%).

Table 1. Demographic and clinical characteristics of the patients

Characteristics	Mean±SD	Median (min-max)
Age (year)	48.85±12.72	48.5 (23–79)
Sex, n (%)		
Male	36 (76.6)	
Female	11 (23.4)	
Duration of treatment (day)	10.96±5.7	11 (3–27)
Leukocytes (cells/mm^3)	23,605.96±12,952.22	21,950 (5,910–63,400)
Neutrophil (%)	89.18±8.2	92.4 (58.9–96.1)
Lymphocytes (%)	5.34±4.85	3.8 (0.5–24.8)
Neutrophil-to-lymphocyte ratio (NLR)	28.45±28.4	24.05 (2.68–192.2)
Aspartate aminotransferase (AST) (u/L)	218.80±714.52	56.5 (21–4,702)
Alanine aminotransferase (ALT) (u/L)	198.41±680.95	59.0 (21–4,569)
Albumin (g/dL)	2.78±0.29	2.7 (2.3–3.4)
Ferritin (mcg/L)	1870.70±1354.65	1381.5 (362–7,841)
D-dimer (ng/mL)	6,794.09±8,802.05	3,310 (340–35,200)
Procalcitonin (ng/mL)	9.92±20.9	1.37 (0.01–100)
C-reactive protein (CRP) (mg/L)	16.72±17.75	14.26 (0.7–115)
IL-6 expression in liver tissue (%)	62.49±18.87	65.0 (25–90)

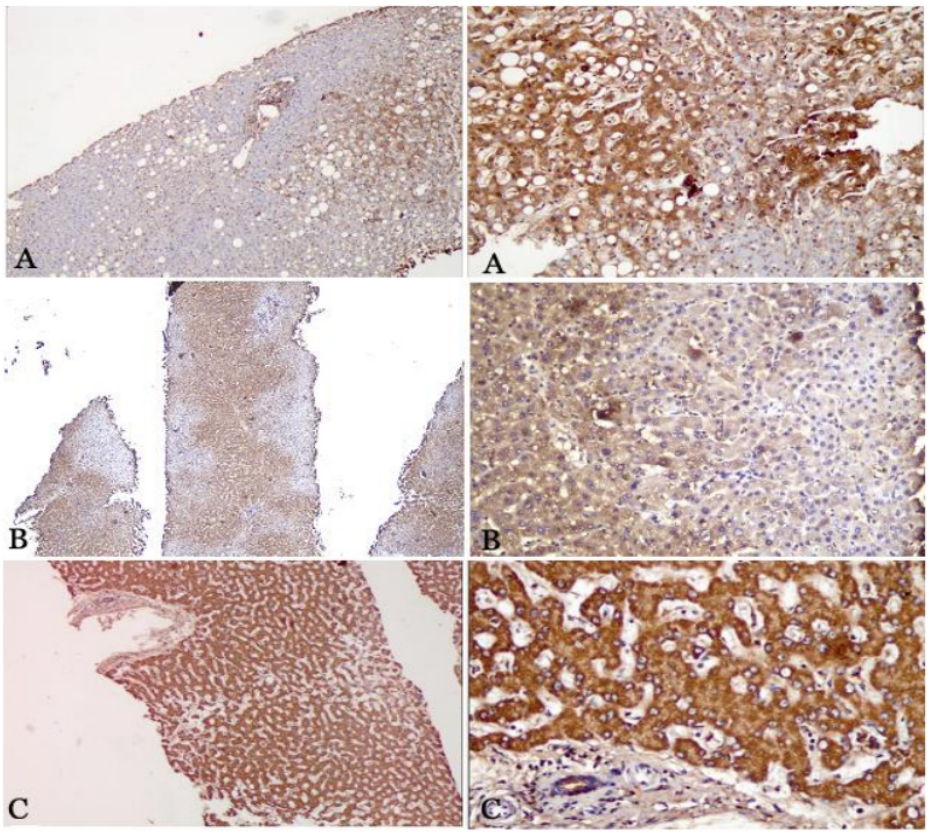

Figure 1. Interleukin 6 (IL-6) expression in patient hepatocytes with slow and higher magnifications for Grade 1 (A), Grade 2 (B) and Grade 3 (C).

Table 2. Interleukin-6 (IL-6) expression in the liver tissue in fatal COVID-19 patients (n=47)

Interleukin-6 expression	Frequency (%)
No expression	0 (0.0)
Positive <30% (mild)	1 (2.1)
Positive 30%–<70% (moderate)	27 (57.4)
Positive ≥70% (strong)	19 (40.4)

IL-6 expression was further characterized based on clinical characteristics, including antiviral and non-antiviral medications, comorbidities, and causes of patients' death (**Table 3**). More than 50% of the patients receiving different antiviral therapies had moderate IL-6 expression, whereas the majority (75.0%) of those receiving tocilizumab (non-antiviral drug) showed strong IL-6 expression. Furthermore, almost half (47.6%) of patients with obesity showed moderate and strong IL-6 expression, and more than half (55.6%) of the patients passing away due to acute respiratory distress syndrome (ARDS) and septic shock had moderate IL-6 expression (**Table 3**).

Table 3. Interleukin-6 (IL-6) expression in fatal COVID-19 patients based on clinical characteristics

Characteristics	Total	<30% n (%)	≥30%–70% n (%)	≥70% n (%)	Mean±SD
Antiviral drug					
Isoprinosine	46	1 (2.2)	26 (56.5)	19 (41.3)	62.43±19.07
Lopinavir-ritonavir	34	0 (0.0)	20 (58.8)	14 (41.2)	63.44 ±18.06
Oseltamivir	4	1 (25.0)	2 (50.0)	1 (25.0)	52.5±27.83
Favipiravir	3	0 (0.0)	2 (66.6)	1 (33.3)	60±27.84
Non-antiviral drug					
Dexamethasone	30	1 (3.3)	21 (70.0)	8 (26.7)	58.3±18.3
Tocilizumab	4	0 (0.0)	1 (25.0)	3 (75.0)	67.5±19.36
Paracetamol and non-steroidal anti-inflammatory drugs	32	1 (3.1)	20 (62.5)	11 (34.4)	60.38±19.30
Comorbidities					
Diabetes mellitus	36	0 (0.0)	22 (61.1)	14 (38.9)	62.47±18.91
Hypertension	27	0 (0.0)	17 (63.0)	10 (37.9)	62.81±18.5
Chronic kidney disease	12	0 (0.0)	5 (71.4)	7 (28.6)	59.71±17.95
Obesity	21	1 (4.8)	10 (47,6)	10 (47.6)	62.52±21.70
Gravida	1	0 (0.0)	1 (100.0)	0 (0.0)	40.0
Hepatitis B	3	0 (0.0)	0 (0.0)	3 (100.0)	61.0±18.56
Causes of mortality					
ARDS and septic shock	45	1 (2.2)	25 (55.6)	19 (42.2)	62.98±18.98
Cardiogenic shock	2	0 (0.0)	2 (100.0)	0 (0.0)	51.5±16.26

Correlation between interleukin 6 (IL-6) expression and liver injury

Almost 60% of the patients experienced liver injury, in which mild injury was found in 23.4%, moderate in 25.5%, and severe in 10.6% of the patients (**Table 4**). The result of the Spearman correlation test suggested no statistically significant correlation between IL-6 expression in liver tissue and the degree of liver injury in fatal COVID-19 patients (r=0.152, p=0.309).

Table 4. Degree of liver injury in fatal COVID-19 patients (n=47)

Degree of liver injury	Frequency (%)
No liver injury	19 (40.4)
Mild	11 (23.4)
Moderate	12 (25.5)
Severe	5 (10.6)

Discussion

COVID-19 is known to trigger a cytokine storm through overactivation of the immune system and uncontrolled release of cytokines. This condition leads to multiorgan injury that results in critical illness and death [16,23]. Liver damage at various degrees has been reported in COVID-19 patients and has been associated with a higher rate of mortality among patients [24]. Elevated levels of IL-6, which induce inflammatory signals, have been widely reported in COVID-19 patients [14,25,26]; however, studies regarding IL-6 expression in the liver tissues and its

association with the degree of liver injury are completely lacking. Therefore, in this study, we examined post-mortem core liver biopsies of deceased COVID-19 patients to identify IL-6 expression in liver tissue and evaluate its correlation with the degree of liver injury. Our immunohistochemical staining revealed the presence of IL-6 expression in the liver tissues of all patients, in which more than half (57.4%) showed moderate, whereas 40.4% exhibited strong IL-6 expression. This suggested an excessive immune activation, likely associated with the occurrence of a cytokine storm.

IL-6 serves as a primary mediator in the acute phase response to infections, and its release into circulation during an acute infection will subsequently stimulate the production of acute-phase proteins by the liver [27]. Thus, high expression of IL-6 in the liver tissues in this study was presumably associated with elevated serum IL-6 levels in COVID-19 patients. Consistent with this immunohistochemical finding, we also found increased levels of several inflammatory markers in the patients, including CRP, ferritin, D-dimer, and procalcitonin, confirming immune system overactivation in fatal COVID-19 patients. Studies found that elevated CRP, procalcitonin, D-dimer, and ferritin levels in COVID-19 patients have been associated with poor prognosis of the disease, including multi-organ failure and mortality [28-30].

Liver dysfunction, recognized by elevated levels of ALT, has been widely reported among patients with fatal COVID-19 [7,31]. The liver injury was caused by inflammation and damage by SARS-CoV-2, marked by elevated of ALT and AST levels. Due to the similar nature with hepatitis, this liver injury was also suggested as coronaviral hepatitis or COVID-19 induced hepatitis (CIH) [8,32]. We found that more than half of the patients experienced liver injury, ranging from mild to severe. Similar to our finding, a previous study also reported abnormal AST and ALT levels in more than half of COVID-19 cases during the disease progression [7]. The prevalence of liver injury was higher in patients with fatal COVID-19 than those with moderate infection [31,33]. Furthermore, we also observed decreased levels of serum albumin among the patients, indicating not only impaired liver function but also a poor prognosis of the disease. Patients with normal albumin levels have been reportedly at a lower risk of mortality as compared to those with lower albumin levels [34,35].

Despite both IL-6 expression in the liver and increased serum ALT have been observed among fatal COVID-19 in the present study, the result of the correlation analysis indicated an insignificant correlation between IL-6 expression and the degree of liver injury (r=0.152 with p=0.309). This might suggest that IL-6 is not the only inflammatory factor contributing to liver injury in COVID-19 patients in this study. To date, no study has been carried out related to IL-6 expression in the liver tissue of COVID-19 patients through immunohistochemical staining, thus comparison of our study to others could not be performed. Several reported investigations focused mainly on serum or systemic IL-6 and other inflammatory markers and their association with liver injury in COVID-19 patients [9-11,36,37]. Other proinflammatory cytokines, besides IL-6, have been explored in COVID-19 patients and are typically linked to significant lung injury, including IL-1, IL-7, IL-12, interferon (IFN), tumor necrosis factor (TNF), macrophage inflammatory protein-1 alpha (MIP-1A), monocyte chemoattractant protein-1 (MCP-1), granulocyte-colony stimulating factor (GCSF), and interferon-inducible protein-10 (IP-10) [38]. Meanwhile, their contribution to liver damage is currently unclear. Not many studies examine the association between SARS-CoV-2 infection and inflammatory response, as well as the expression of cytokines like TNF, IL-1, IL-6, MCP-1, IL-15, and leukocyte markers (CD 4, CD 8, CD20, and CD 45), in an effort to confirm and define the role and expression of cytokines, particularly their effects on liver tissue [38]. According to a study, elevated levels of CRP, TNF, and IL-6 were linked to a higher risk of the severe COVID-19, which can cause liver damage [39].

In this study, other inflammatory markers, including CRP, serum ferritin, D-dimer were also significantly elevated. This result raised the possibility that these markers could also be used to identify liver injury in COVID-19 patients who are critically ill. COVID-19-associated liver injury in fact involves complex mechanisms. The lack of significance between IL-6 expression and the severity of liver damage in this present study may be due to other mechanisms that predominate in liver damage. In addition to systemic inflammation due to cytokine storms, other factors such as direct cytopathic effect of viral invasion, immune reconstitution due to the presence of SARS-CoV-2, drug toxicity, or liver hypoxia due to COVID-19 pneumonia might also suggestively

contribute to liver injury in COVID-19 patients [7,40-41]. The process of inflammation or damages in hepatocytes as a result of interaction between Kupffer cells and cytotoxic T-cell in which causing another condition that is similar with viral hepatitis [8]. In this study, patients with moderate and strong IL-6 expression in the liver tissue were treated with various antiviral and non-antiviral medications, including lopinavir/ritonavir, and anti-IL-6 (tocilizumab), leading to an assumption these drugs might play a role in hepatic injury induction among the patients [41]. Elevated CRP and NLR, which were also observed in the patients are also among potential risk factors for liver injury and COVID-19 severity [31,42].

This study possessed several limitations that should be addressed. The study was performed among fatal COVID-19 infections, in which their comorbidities at the time of admission or during treatment might serve as a confounding factor affecting the results. Furthermore, this study did not exclude the use of hepatotoxic drugs for COVID-19 therapy, which may contribute to liver injury in COVID-19 patients. Therefore, eliminating any plausible confounding factors influencing the results in future studies should be put into consideration.

Conclusion

We conducted a cross-sectional study among fatal COVID-19 patients to evaluate the association of IL-6 expression in the liver tissues with the degree of liver injury. All post-mortem core liver biopsies were positive for IL-6 immunohistochemical staining; and liver injury at various degree was observed in more than half of samples. However, the result showed no statistically significant correlation between IL-6 expression in the liver and the degree of liver injury among patients with fatal COVID-19, suggesting that IL-6 expression in the liver tissue was not the only parameter for the determination of liver injury in critically ill COVID-19 patients. Further investigations involving a larger number of samples with a wider range of COVID-19 severity, numerous cytokines, inflammatory cells, and markers, are required to obtain more accurate results.

Ethics approval

This study was approved by the Research Ethics Committee of Dr. Soetomo General Academic Hospital, Surabaya, Indonesia (No. 0022/KEPK/VII/2020).

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

How to cite

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