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Analysis of natural resistance-associated macrophage protein-1 (NRAMP-1) level based on death, comorbidities and severity of COVID-19 patients: a cross-sectional study

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Background: An accurate diagnosis of COVID-19 is essential for pandemic control and for establishing adequate therapeutic strategies to reduce morbidity and mortality. COVID-19 infection replicates in macrophage cells and affects the immune system. Natural resistance-associated macrophage protein-1 (NRAMP-1) carries cation ions, such as $Fe2+$, $Zn2+$ and $Mn2+$, and plays an essential role in the immune system to infection with micro-organisms. In addition, the function of NRAMP-1 is to limit the replication of pathogens by changing the phagosomal environment. Levels of NRAMP-1 protein are based on death, comorbidities and clinical symptoms of COVID-19 patients and it is possible for the soluble protein NRAMP-1 level to be used as an additional biomarker for forensic and medicolegal related COVID-19 cases and prosecutions from patients and families.

Methods: Determination of NRAMP-1 protein levels using the enzyme link-immunosorbent assay technique in death, had comorbidities and severity of clinical symptoms of COVID-19 patients.

Results: Of the 62 patients who received treatment, 10 patients died with an average NRAMP-1 level of 650 ng/ml and 52 patients who survive with an average NRAMP-1 level of 1065.26 ng/ml. The results of the study also found that 34 patients had comorbidities with an average NRAMP-1 level of 838.82 ng/ml and 28 patients without comorbidities with an average NRAMP-1 level of 1191.92 ng/ml. Based on the severity of clinical symptoms in survive patients, 10 patients with mild were found with an average NRAMP-1 level of 984.31 ng/ml, with moderate in 31 patients with an average NRAMP-1 level of 1104.71 ng/ml and severe in 11 patients with an average NRAMP-1 level of 1027.71 ng/ml.

Conclusions: NRAMP-1 protein levels were significantly lower in COVID-19 patients who died and had comorbidities.

Keywords: Comorbidities, COVID-19, death, ELISA, NRAMP-1, severity

Introduction

At the beginning of 2020, the world was shocked by the outbreak of a new virus, namely a new type of coronavirus (SARS-CoV-2)

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HIGHLIGHTS

- Natural resistance-associated macrophage protein-1 (NRAMP-1) protein levels were significantly lower in COVID-19 patients who died, had comorbidities and severe of clinical symptoms.
- The NRAMP-1 protein levels can be used as a potential additional biomarker in diagnosing the mortality, comorbidities and severity of clinical symptoms of COVID-19 patients.
- Further research is needed longitudinally to look at the dynamics of NRAMP-1 values in the course of the COVID-19 disease from the time of hospital admission to hospital discharge.
- There is also a need for multicentre studies to determine protein levels of NRAMP-1 as a biomarker of COVID-19 in mortality, comorbidities and severity of clinical symptoms associated with clinical forensic and medicolegal cases.

and the disease is called COVID-19. It is known, the origin of this virus originated from Wuhan, China. It was discovered at the end of December 2019. Until now it has been confirmed that there are 65 countries that have contracted this one virus[1].

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Based on data up to 2 March 2020, the worldwide mortality rate is 2.3%, while specifically in the city of Wuhan it is 4.9%, and in Hubei province it is 3.1%. This figure in other provinces in China is 0.16%. Based on a study of the first 41 patients in Wuhan, six people died (five patients in ICU and one patient non-ICU)[2]. Many cases of death in the elderly and with comorbidities. The first case of death was a 61-year-old male patient with comorbid intra-abdominal tumours and abnormalities in the liver[3]. Previous study revealed that a significant relationship between the D-dimer and prothrombin time parameter in confirmed COVID-19 in patients and hypertension and diabetes mellitus are significantly correlated with (CRP), C-Reactive Protein level. Chest X-ray finding is also significantly correlated with C-Reactive Protein level in COVID-19 patients[4,5].

Natural resistance-associated macrophage protein-1 (NRAMP-1) is a gene found in mammals and has an effects on the immune system. NRAMP-1 is a gene in intracellular macrophages located on the phagosomal membrane on chromosome 2q35. NRAMP-1 functions as a carrier for cation ions namely $Fe2+$, $Zn2+$ and $Mn2+$ and plays an important role in the immune system against infection by micro-organisms and elimination of bacteria and viruses. Besides that, the function of NRAMP-1 is to limit pathogen replication by changing the phagolysomal environment[6].

The levels of NRAMP-1 protein based on the mortality, comorbidities and severity of COVID-19 patients, it is possible to be used as an additional biomarker for COVID-19 patients related to forensic and medicolegal cases and also, concerning with a prosecution of patients and their families.

Thus, there are needed in conducting research on NRAMP-1 protein levels based on mortality, comorbidities and severity of clinical symptoms in patients with COVID-19.

Materials and methods

COVID-19 patients and sample collection

This research is a cross-sectional study. The sample used is the blood of a COVID-19 patients. The NRAMP-1 protein levels were examined using the enzyme link-immunosorbent assay (ELISA) technique.

The inclusion criteria of the patients are COVID-19 patients who are hospitalized with complete clinical data with positive COVID-19 polymerase chain reaction (PCR) results.

The severity of the patient is the clinical, laboratory and radiological symptoms found in confirmed COVID-19 patients and is determined in the clinical symptoms of mild, moderate, severe and critical degrees[1,7].

Blood samples from COVID-19 patients that have been taken will be subject to several tests using the PCR and ELISA technique. Swap test to determine positive COVID-19 patients and diagnosis of COVID-19 by real-time PCR[4,5]. Determination of NRAMP-1 protein levels in subjects by ELISA technique[8–11].

Data collection was carried out by the main researcher and assisted by enumerators. In this study, the authors confirmed that all methods were carried out following the relevant guidelines and regulations of Ethical Committee of Hasanuddin University, Makassar, Indonesia, date 27 September 2022, with Number 545/UN.4.6.4.5.31/PP36/2022.

This manuscript has been reported in line with the STROCSS 2021 criteria[12].

This study is not experimental, not intervention research and not multicentre study.

Determination of NRAMP-1 protein levels in COVID-19 patients using the ELISA

Serum obtained from the required sample was removed from the freezer − 20ºC and stored on ice before use. Each sample was duplicated to ensure the validity of the ELISA results. The first stage was the addition of 100 μl assay diluent containing protein buffer into each well. Then add 100 μl of standard fluid containing the target recombinant human protein NRAMP-1 from the specified kit or sample dilution from the patient's serum into each well. Then incubated for 2 h at room temperature. Suck the liquid in each well and wash with sterile (PBS), Phosphate-buffered saline. This washing process was carried out four times in succession. Then 200 μl of conjugate liquid containing strep-tavidin (HRP), Horseradish Peroxidase was added to each well/well and covered with a plastic cover and incubated for 2 h at room temperature. The liquid was sucked in and then washed again 4 times using sterile Phosphate-buffered saline. In the next process, 200 μl substrate Solution containing 3,3',5,5' (TMB), tetramethylbenzidine was added to each well and read using an ELISA Reader 270 (Biomerieux, France)[13–15].

Statistical analysis

All the data obtained were grouped according to the purpose and type of data, then the appropriate statistical method was selected and processed using software SPSS version 23 (computerized statistical software) and then layer analysis was carried out. The statistical analysis technique using ANOVA test was used to compare numerical difference in each group. paired t-test and independent t-test was used to compare the NRAMP-1 protein levels of each COVID-19 patient group, before and after experiment. P value less than 0.001 was considered significant.

Results

Table 1. Shows the characteristics of COVID-19 patients. It shown that from a total of 62 patients, 22 were male (35.48%) with an average value of NRAMP-1 levels is 982 ng/ml and female 40 (64.52%) with an average value of 1007 ng/ml NRAMP-1 protein level. The total mean value of NRAMP-1 levels is 998 ng/ml with a standard deviation of 331, the minimum value for NRAMP-1 is 373.7 ng/ml and the maximum value for NRAMP-1 is 1493 ng/ml. The average age is 52 years with a standard deviation of 15 years, the minimum age is 18 years and the maximum age is 87 years. For the average value of the Cycle Threshold (Ct Value) in patients, it is 24 with a minimum value of 19 and a maximum of 38.5.

The results of the study found 28 patients without comorbidities (45.2%) with an average NRAMP-1 value of 1191.92 ng/ml. There were 9 (14.5%) hypertensive patients with an average NRAMP-1 value of 838.89 ng/ml. Patients with heart disease were 1 (1.6%) with an average value of NRAMP-1 is 1199 ng/ml. Patients with a history of thyroid surgery 1 (1.6%) with an average value of NRAMP-1 is 476 ng/ml. Patients with hypertension and heart disease 1 (1.6%)with an average NRAMP-1 value of 1226.7 ng/ml. Patients with coronary artery disease (CAD) and heart disease 3 (4.8%) with an average NRAMP-1

Table 1

Characteristic of COVID-19 patients.

Variable	Number ($n = 62$)	(%)	Mean NRAMP-1 (ng/ml)
Sex			
Male	22	35.48	982.13
Female	40	64.52	1007.17
Age (mean ± SD;min–max) year	52.00 ± 15.0770 (min 18–max 87)		
Ct (mean ± SD;min–max)	24.4863 ± 6.10625 (min 10.56–max 38.50)		
NRAMP-1 (mean ± SD;min–max)	998,29 ± 331,30 (min 373,72–max 1492.78)		
Comorbidities			
No	28	45.2	1191.92
HT	9	14.5	838.89
DM	5	8.1	880.07
Hyperthyroidism	1	1.6	476.08
A	1	1.6	1199.37
HT + A	1	1.6	1226.66
CAD	3	4.8	784.96
Diabetes Mellitus + HT	8	12.9	694.16
DM + CAD + HT	1	1.6	1472.31
HT + CAD	1	1.6	970.78
HT + BA	1	1.6	1015.13
DM + S + HT	1	1.6	938.6
DM + HK	1	1.6	578.79
DM + HT + CAD	1	1.6	783.91
Clinical symptoms			
Death	10	16.1	649.99
Fever	47	75.8	1006.57
Cough	47	75.8	987.06
Like common cold	13	21.0	1052.79
Apnoea	34	54.8	962.82
Nausea	35	56.5	1035.64
Heartburn	15	24.2	1042.18
Vomiting	15	24.2	1181.6
Headache	17	27.4	1104.77
Delirium	1	1.6	476.08
Diarrhoea	14	22.6	1175.93
Weakness	34	54.8	988.55
Loss of taste	2	3.2	1158.42
Loss of smell	2	3.2	1106.35
Reduce appetite	16	25.8	1202.67

A, arrhythmia; BA, brochial asthma; CAD, coronary artery disease; Ct, cycle threshold; DM, diabetes mellitus; HK, hypokalemia; HT, hypertention; Max, maximum; Min, minimum; NRAMP-1, natural resistance-associated macrophage protein-1; S, stroke.

value of 785 ng/ml. Patients with diabetes mellitus and hypertension 8 (12.9%) with an average NRAMP-1 value of 694 ng-/mL. Patients with cardiovascular disease, diabetes mellitus and hypertension were 1 (1.6%) with an average NRAMP-1 value of 1472 ng/ml. Patients with CAD and hypertension were 1 (1.6%) with an average value of NRAMP-1970.78 ng/ml. Patients with hypertension and bronchial asthma were 1 (1.6%) with an average NRAMP-1 value of 1472 ng/ml. Patients with CAD and Hypertension were 1 (1.6%) with an average NRAMP-1 value of 1015 ng/ml. Patients with diabetes mellitus, stroke and hypertension were 1 (1.6%) with an average value of NRAMP-1938.6 ng/ml. Patients with diabetes mellitus and chronic hypokalaemia were 1 (1.6%) with an average value of NRAMP-1 is

578.79 ng/ml. Patients with diabetes mellitus and chronic hypokalaemia were 1 (1.6%) with an average value of NRAMP-1578.79 ng/ml.Patients with CAD, diabetes mellitus and hypertension were 1 (1.6%) with an average value of NRAMP-1 is 784 ng/ml.

Based on clinical symptoms, 10 patients died during treatment (16.1%) with an average value of NRAMP-1 is 650 ng/ml. Patients with clinical symptoms of fever were 47 (76%) with an average value of NRAMP-1 is 1006.57 ng/ml. Patients with clinical symptoms of cough as many as 47 (75.8%) with an average value of NRAMP-1 is 987 ng/ml. Patients with clinical symptoms of common cold as many as 13 (21%) with an average value of NRAMP-1 is 1053 ng/ml. Patients with clinical symptoms of shortness of breath were 34 (54.8%) with an average value of NRAMP-1 is 962.8 ng/ml. Patients with symptoms of nausea as many as 35 (56.5%) with an average value of NRAMP-1 is 1035.6 ng/ml. Patients with symptoms of heartburn as many as 15 (24.2%) with an average value of NRAMP-1 is 1042.2 ng/ml. Patients with symptoms of vomiting as many as 15 (24.2%) with an average value of NRAMP-1 is 1182 ng/ml. Seventeen patients with headache symptoms (27.4%) with an average value of NRAMP-1 is 1104.8 ng/ml. Patients with clinical symptoms decreased level of consciousness by 1 (1.6%) with an average value of NRAMP-1 is 476.1 ng/ml. Patients with clinical symptoms of diarrhoea were 14 (22.6%) with an average value of NRAMP-1 is 1176 ng/ml. Patients with symptoms of weakness as many as 34 (55%) with an average value of NRAMP-1 is 989 ng/ml. Patients with clinical symptoms of loss of taste as much as 2 (3.2%) with an average value of NRAMP-1 is 1158 ng/ml. Patients with clinical symptoms of loss of smell as much as 2 (3.2%) with an average value of NRAMP-1 is 1106 ng/ml. Patients with clinical symptoms decreased appetite by 16 (26%) with an average value of NRAMP-1 is 1207 ng/ml.

Table 2 shows a comparison of NRAMP-1 protein levels based on patients who died, degree of severity disease, where the total number of patients who were admitted to the hospital with symptoms of COVID-19 and positive PCR swab examination results were obtained as many as 62 patients. Of the 62 patients who received treatment, there were 10 patients who died with an average NRAMP-1 level of 650 ng/ml and 52 patients who lived with an average NRAMP-1 level is 1065.26 ng/ml. The NRAMP-1

Table 2

The comparison of NRAMP-1 protein level in death, comorbidities and severity of COVID-19 patients.

No	Variable	Mean of NRAMP-1 (ng/ml)	P
1	Death ($n = 10$)	649.99	< 0.001
	Survive ($n = 52$)	1065.26	
2	Comorbidities		< 0.001
	Yes ($n = 34$)	838.82	
	No ($n = 28$)	1191.92	
3	Degree of severity in survive patients		
	Mild ($n = 10$)	984.31	a. > 0.001*
	Moderate ($n = 31$)	1104.71	b. > 0.001**
	Severe ($n = 11$)	1027.71	c. > 0.001***

NRAMP-1, natural resistance-associated macrophage protein-1.
*a. Mild vs. moderate.
**b. Moderate vs. severe.
***c. Mild vs. severe.

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protein levels of dead patients were significantly lower compared to the survive of COVID-19 patients ($P < 0.001$).

The results of the study also found that 34 patients had comorbidities with an average NRAMP-1 level of 838.82 ng/ml and 28 patients without comorbidities with an average NRAMP-1 level of 1191.92 ng/ml. The NRAMP-1 protein levels of had comorbidities patients were significantly lower compared to the without comorbidities of COVID-19 patients ($P < 0.001$).

Based on the severity level (survive patients), 10 patients with mild symptoms were found with an average NRAMP-1 level of 984.31 ng/ml, with moderate symptoms in 31 patients with an average NRAMP-1 level of 1104.71 ng/ml and severe symptoms in 11 patients with an average NRAMP-1 level of 1027.71 ng/ml. The NRAMP-1 protein levels between mild, moderate and severe of COVID-19 patients were not significantly different ($P > 0.001$).

Discussion

Based on the results of the study, the distribution of patients found that more female were found with COVID-19 than male. This is in line with the results of previous study conducted in Germany and it was found that female are more exposed to COVID-19 as a result of specific close contact higher among female. In previous study, an exploration was carried out on the close contact behaviour of the sex that affected COVID-19 infection and death. The purpose of this study was to establish short-term predictions of the epidemic that occurred during COVID-19 within 75 days, where there were differences based on age group, sex, estimated contact patterns and social behaviour. To further explore the possible paths, more data is needed on contact behaviour and transmission of COVID-19, which includes sex and socio-demographic information. The study revealed that female make the most social contact such as shopping, going on vacation trips and others which are the main cause of the high cases of COVID-19 in female[3].

From the results of research that has been done, it was found NRAMP-1 protein levels were significantly lower in COVID-19 patients who died compared with those who were survive. Where it can be seen in Table 2, the comparison between NRAMP-1 protein levels in patients who died was much lower than survive patients. The results of this study are supported by the theory that NRAMP-1 produced by macrophage cells functions to increase the immune system in infectious diseases, so that low NRAMP-1 levels are closely related to the death of COVID-19 patients. The NRAMP-1 protein associated with macrophage activity, consists of 548 amino acids and 12 transmembrane domains localized in the lysosomal membrane. Overall, NRAMP-1 comprises several targeting motifs for function into lysosomes that function in the body's overall immune system. NRAMP-1 protein levels were significantly lower in COVID-19 patients who had comorbidities than those without comorbidities. This can be caused because the presence of comorbidities will reduce the function of macrophages which are closely related to the NRAMP-1 protein in reducing the immune system in infectious diseases with other comorbidities diseases[16].

The results of this study found that comorbidity determines the severity of the patient's disease, where the presence of comorbidities will likely reduce the function of macrophages and other immune systems in eliminating viruses. In a previous study, 76.7% of patients who were infected with COVID-19 in the Detroit metropolitan area were hospitalized. The high prevalence of comorbidities and obesity in COVID-19 patients may contribute to the severity and mortality of COVID-19 patients. In COVID-19 patients with comorbidities, high complications and death were found in the ICU. Complication rates, including acute renal failure, hypoxia, required ventilators and prolonged hospitalization. Overall, deaths in the ICU were higher than deaths in the ward. And significantly the most deaths were found in COVID-19 patients with male over 60 years of age[17].

NRAMP-1 function on macrophages, so that decreased NRAMP-1 function will cause severity accompanied by comorbidities in patients with COVID-19. In previous studies it was found that patients with COVID-19 can experience high regulation of cytokine production, especially in patients who are in critical condition accompanied by pneumonia. Of the 41 COVID-19 patients, most of the patients experienced mild symptoms, while some patients showed symptoms that were getting more severe and ended in death due to multiple organ dysfunction syndrome (MODS). MODS is caused by a severe cytokine storm and it is necessary to monitor cytokine levels in every patient with COVID-19. Where this cytokine storm is an important component as a cause of death in COVID-19 disease. Some COVID-19 patients will experience systemic inflammatory response syndrome and MODS which are characterized by uncontrolled release of inflammatory mediators, causing a cytokine storm that contributes to increased mortality in acute respiratory distress syndrome[18].

Because the recommendation not to perform autopsies for COVID-19 is still controversial to a certain degree, but from a medicolegal aspect is an important role that autopsy may play in gaining knowledge of many aspects of the disease[19]. Thus, needed a potential biomarker which easier and more comfortable, and acceptable for COVID-19 patients and their families.

The overall results of this study indicate that NRAMP-1 protein levels can be used as a potential additional biomarker in diagnosing the mortality, comorbidities and severity of clinical symptoms of COVID-19 patients. The limitation of this study is that the mRNA expression of the NRAMP-1 gene was not examined in stages. Thus, further research is needed longitudinally to look at the dynamics of mRNA NRAMP-1 expression in the course of the COVID-19 disease from the time of hospital admission to hospital discharge. And there is also a need for multicentre studies to determine protein levels of NRAMP-1 as a biomarker of COVID-19 mortality, comorbidities and severity of clinical symptoms associated with clinical forensic and medicolegal cases.

Conclusion

There is a strong relationship between NRAMP-1 protein levels and mortality, comorbidities, but not in severity of clinical symptoms in patients with COVID-19. Further cohort studies are needed to determine the dynamics of NRAMP-1 levels according to the course of the disease in patients with COVID-19.

Ethical approval

This research has passed the ethical test based on the research ethics notification letter from the Faculty of Medicine,

Hasanuddin University, Makassar, Indonesia date 27 September 2022, with Number 545/UN.4.6.4.5.31/PP36/2022.

Consent for publication

Written informed consent was obtained from the patient or the patient's family for the publication of this study.

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Author contribution

R.S., A.A.M., M.H., D.A., G.A., B.J.N., M.H.C., G.S.L. and J.D. conceived and designed the study, conducted research, provided materials and collected and organized data. R.S., M.H., D.M., I.A.R., C.K., A.E., A.R.J., D.A., A.F., A.A. and A.S. drafted the manuscript. R.S., A.S., M.R.P., A.A., M.F., A.R.J., A.F. and M.H. analyzed the data and interpreted data. R.S., A.S., A.A., M.H.C., A.R.J., A.F., M.F. and M.H. wrote initial and final draft articles, and provided logistical support. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

Conflicts of interest disclosure

The authors declare no conflict of interest, financial or otherwise.

Research registration unique identifying number (UIN)

This study is not experimental, and not intervention research and not multicentre study. This research has passed the ethical test based on the research ethics notification letter from the ETHICS COMMITTEE, Faculty of Medicine, Hasanuddin University on September 27, 2022, with Number 545/UN.4.6.4.5.31/PP36/ 2022. Data collection was carried out by the main researcher and assisted by enumerators. In this study, the authors confirmed that all methods were carried out following the relevant guidelines and regulations of ETHICS COMMITTEE of Faculty of Medicine, Hasanuddin University, Makassar, Indonesia

Guarantor

Prof. Mochammad Hatta.

Data availability statement

Datasets generated during and/or analyzed during the current study are publicly available.

Provenance and peer review

Not commissioned, externally peer-reviewed.

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