Discussion about clinical value of detection of IL-10, IL-1β, IL-6, MCP-1, TNF-α, IP-10 and IL-4 for the diagnosis of COVID-19

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July 1, 2020

Abstract

Background and purpose: Studies have shown that some cytokines of COVID-19 were elevated. This study aims to assess whether IL-10, IL-1β, IL-6, MCP-1, TNF-α, IP-10 and IL-4 serve as diagnostic biomarkers of COVID-19 and offer prognostic insight upon initial presentation to help guide treatment,In addition, the relationships between them and gender, age, antibody concentration and course of disease were also discussed. Methods: The serum levels of cytokines above in experience group (COVID-19 patients) and control group (other diseases patients and healthy people) were detected by ELISA. Results: Most of the serum level of cytokines above in experience group were significantly higher than those in control group, and AUCs of COVID-19 diagnosed by they were 0.735, 0.775, 0.595, 0.821, 0.848, 0.387 and 0.987. The serum levels of some cytokines in male patients had noticeably higher than those in female patients, while the serum levels of almost all cytokines of the elderly were higher than that of the youth and middle-aged patients. The serum levels of IP-10 in patients were positively correlated with IgM, while TNF-α were negatively correlated with IgG. The levels of IL-1 β and IL-6 increased sharply in the early stage of COVID-19, then decreased gradually; the levels of IL-10, MCP-1, TNF-α and IL-4 increased sharply in the middle stage, while the levels of IP-10 increased sharply in the late stage. Conclusion: The cytokines above can prove to be great significance for clinical diagnostics of COVID-19, and the levels of cytokines in patients have some relationships with gender, age and course of disease.

Δισϛυσσιον αβουτ ϛλινιϛαλ αλυε οφ δετεϛτιον οφ ΙΛ-10, ΙΛ-1β, ΙΛ-6, MˆΠ-1, ΤΝΦ-α, ΙΠ-10 ανδ ΙΛ-4 φορ τηε διαγνοσις οφ ˆO˝ΙΔ-19

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Abstract: Background and purpose : Studies have shown that some cytokines of COVID-19 were elevated. This study aims to assess whether IL-10, IL-1β, IL-6, MCP-1, TNF-α, IP-10 and IL-4 serve as diagnostic biomarkers of COVID-19 and offer prognostic insight upon initial presentation to help guide treatment, in addition, the relationships between them and gender, age, antibody concentration and course of disease were also discussed. **Methods:** The serum levels of cytokines above in experience group (COVID-19 patients) and control group (other diseases patients and healthy people) were detected by ELISA. **Results:** Most of the serum level of cytokines above in experience group were significantly higher than those in control

group, and AUCs of COVID-19 diagnosed by they were 0.735, 0.775, 0.595, 0.821, 0.848, 0.387 and 0.987. The serum levels of some cytokines in male patients had noticeably higher than those in female patients, while the serum levels of almost all cytokines of the elderly were higher than that of the youth and middle-aged patients. The serum levels of IP-10 in patients were positively correlated with IgM, while TNF-α were negatively correlated with IgG. The levels of IL-1 β and IL-6 increased sharply in the early stage of COVID-19, then decreased gradually; the levels of IL-10, MCP-1, TNF-α and IL-4 increased sharply in the middle stage, while the levels of IP-10 increased sharply in the late stage.**Conclusion:** The cytokines above can prove to be great significance for clinical diagnostics of COVID-19, and the levels of cytokines in patients have some relationships with gender, age and course of disease.

Keywords: cytokine; COVID-19; clinical value; course of disease;

Q: What's already known about this topic?

A: Symptoms of COVID-19 patients, infection pathways of SARS-CoV-2, and the COVID-19 patients have higher cytokines**.**

Q: What does this article add?

A: What kinds of cytokines of COVID-19 patients were increased, whether they serve as diagnostic biomarkers of COVID-19 and offer prognostic insight upon initial presentation to help guide treatment, their relationship with age, gender,antibody concentration and course of disease were also discussed.

Coronavirus disease 19 (COVID-19) was first discovered in Wuhan, Hubei in December 2019 [1], then the epidemic spread throughout China and other countries and regions abroad[2]. The virus has high infectivity and caused serious damage to the global economy and the health of people all over the world. The main methods for diagnosing novel coronavirus (SARS-CoV-2) were nucleic acid detection and serological antibody detection [3]. According to diagnosis and treatment plan of COVID-19 (trial version 7) issued by the office of the Chinese health and Health Commission and the office of the State Administration of traditional Chinese medicine, inflammatory factors are often increased in severe and critical patients[4]. The immune cells can secrete cytokines (CKs) such as interleukin (IL), colony-stimulating factor (CSF), chemokine, interferon (IFN), tumor necrosis factor (TNF) and growth factor (GF) which can induce the activation, proliferation or migration of target cells by binding to specific receptors on a variety of cells when the body was immune to exogenous substances [5]. Our study aims to detect the expression level of IL-10, IL-1β, IL-6, monocyte chemoattractant protein-1 (MCP-1), TNF-α, interferon-inducible protein -10 (IP-10) and IL-4 in the blood of in COVID-19 patients, other diseases patients and healthy people, and discuss its diagnostic value for diagnosis of COVID-19.

Materials and methods

1.1 Materials

1.1.1 Specimens

120 experience group blood samples were from 48 COVID-19 patients (confirmed by positive nucleic acid test, including 2 dead patients, 17 asymptomatic infected patients) in Loudi Center for Disease Control and Prevention, time from onset to discharge of 3 of other 29 patients were unknown, of the other 26 patients were shown in Figure 1; 88 control group blood samples were from The First Hospital of Hunan University of Chinese medicine (53 patients with malignant tumor, blood system disease, rheumatic immune system disease and other diseases that increase the level of inflammatory factors, and 35 healthy people). 75 experience group blood samples were from males and 35 from females, the age distribution of COVID-19 patients we collected were shown in Figure 2. All the selected cases and their families had given their informed consent.

Instruments and reagents

IL-10, IL-1β, IL-6, MCP-1, TNF-α, IP-10 and IL-4 test kits and their standard were purchased from Human diagnostic products (Beijing) Co., Ltd., the minimal detectable concentrations for they were 0.0225,

0.0355, 0.0600, 0.0655, 0.0386, 0.0102 and 0.1800 pg/mL, count the result which lower than the minimal detectable concentrations as half of the minimal detectable concentrations; all reagents had both good repeatability (CV < 10%) and specificity. TECAN 200-8 were purchased from TECAN (Shanghai) trading company. SAS-CoV-2 antibody test kit (colloidal gold immunochromtographic assay, Guangzhou Wanfu Biotechnology Co., Ltd.) and SAS-CoV-2 IgM and IgG antibody test kit (chemiluminescent immunoassay, purchased from Shenzhen Yahuilong Biotechnology Co., Ltd.) were selected for serum antibody test.

1.2 Methods

The serum samples of all cases were venous blood of 12 hours fasting without hemolysis or hyperlipidemia. The serum was obtained by centrifugation at 4000 RPM for 10 minutes. The serum contents of IL-10, IL-1β, IL-6, MCP-1, TNF-α, IP-10 and IL-4 were detected by double-antibody sandwich ELISA, and a hole was added as a blank control, the samples whose OD value exceeds the linear range should be diluted before detection. The experiment was conducted in strict accordance with the instructions of the kit.

1.3 Statistical analysis

All datas were processed by IBM SPSS statistic 21 and were drawed by GraphPad Prism 7. According to the characteristics of data distribution, independent-samples Mann-Whitney U-test was utilized to compare the serum levels of IL-10, IL-1 β, IL-6, MCP-1, TNF - α, IP-10 and IL-4 in different groups (COVID-19 patients, other diseases patients and healthy people);independent-samples Levene T-test was utilized to compare COVID-19 patients in different genders and ages, the difference was statistically significant with unilateral P-value < 0.05; Pearson Correlation test was utilized to analyze the correlation between the levels of cytokines and IgG and IgM, the difference was statistically significant with bilateral P-value < 0.05.

2 Results

2.1 Δετεςτιον οφ ΙΛ-10, ΙΛ-1β, ΙΛ-6, ΜΆΠ-1, ΤΝΦ-α, ΙΠ-10 ανδ ΙΛ-4 ιν σερυμ σαμπλες οφ ΆΟΎΙΔ-19 πατιεντς, οτηερ δισεασες πατιεντς ανδ ηεαλτηψ πεοπλε βψ ΕΛΙΣΑ

After statistical analysis, as Figure 3 shows, it was found that the level of IL-10, IL-1β, IL-6, MCP-1, TNF-αand IL-4 in the serum of COVID-19 patients were significantly higher than that in the serum of healthy people (P = 0.000), while the serum levels of IP-10 between COVID-19 patients and healthy people were not significant different (P = 0.310); the serum levels of IL-10, IL-1β, MCP-1, TNF-α, IP-10 and IL-4 in COVID-19 patients were significantly higher than those in other diseases patients (P = 0.000, 0.004), while the serum levels of IL-6 between COVID-19 patients and other diseases patients were not significant different (P = 0.078).

2.2 Διαγνοστις εφφιςαςψ οφ δετεςτιον οφ τηε ΙΛ-10, ΙΛ-1β, ΙΛ-6, ΜΆΠ-1, ΤΝΦ-α, ΙΠ-10 ανδ ΙΛ-4 ον ΆΟΎΙΔ-19 βψ ΕΛΙΣΑ

The ability of prediction of IL-10, IL-1β, IL-6, MCP-1, TNF-α, IP-10 and IL-4 on COVID-19 were assessed by ROC curve, as Figure 4 shows, the experimental group (COVID-19 patients) were positive, and the control group (other diseases patients and healthy people) were negative, and select the tangent point with the largest Youden'sindex as the cut-off value. When select 2.621, 4.898, 1.730, 19.948, 0.110, 7.083 and 0.085 pg / mL as their cut-off values respectively, AUCs of COVID-19 diagnose by they were 0.735, 0.775, 0.595, 0.821, 0.848, 0.387 and 0.987, respectively. The sensitivity of diagnosis of IL-4, TNF-α, MCP-1, IL-1β, IL-10 and IL-6 were 99.1%, 81.2%, 84.6%, 63.2 %, 65.8 % and 72.6%, and the specificity of they were 98.8%, 93.0%, 69.8%, 95.3%, 89.5% and 64.0%; the sensitivity (parallel experiment) and specificity (series experiment) of combined diagnosis of IL-4 and TNF-α were 99.8% and 99.9%, respectively.

2.3 Τηε ρελατιονσηιπ βετωεεν σερυμ λεελς οφ ΙΛ-10, ΙΛ-1β, ΙΛ-6, ΜΆΠ-1, ΤΝΦ-α, ΙΠ-10 ανδ ΙΛ-4 ωιτη σεξ ανδ αγε ιν ΆΟΎΙΔ-19 πατιεντς

According to the statistical analysis, as Table 1 shows, it was found that the serum levels of IL-10, MCP-1, TNF-α and IL-4 in male COVID-19 patients were markably higher than those in female patients (P=0.017,

0.004, 0.035, 0.012), while the differences of serum levels of IL-1β, IL-6 and IP-10 between male COVID-19 patients and female patients were not markably significant (P=0.057, 0.278,0.085).

Depending on the age distribution of COVID-19 patients we collected, they were divided into adolescents (aged from 8 to 35 years old), middle-aged people (aged from 36 to 59 years old) and elderly people (aged from 60 to 78 years old). According to the statistical analysis, as Table 2 shows, the serum levels of MCP-1 of the elderly were markably higher than those of adolescents and middle-aged patients (P=0.015, 0.000), and the levels of TNF-α and IL-4 in the serum of the elderly were markably higher than those of middle-aged patients (P=0.002, 0.035), but the difference in the levels of IL-10, IL-1β, IL-6 and IP-10 between COVID-19 patients at different ages were not statistically significant (P>0.05).

2.4 Τηε ρελατιονσηιπ βετωεεν λεελς οφ ΙΛ-10, ΙΛ-1β, ΙΛ-6, ΜΆΠ-1, ΤΝΦ-α, ΙΠ-10 ανδ ΙΛ-4 ανδ αντιβοδψ ςονςεντρατιον ιν σερυμ σαμπλες οφ ΆΟΎΙΔ-19 πατιεντς

The correlation analysis of levels of IL-10, IL-1β, IL-6, MCP-1, TNF-α, IP-10 and antibody concentration in serum samples of COVID-19 patients were shown in Table 3. It was found that the levels of IP-10 in COVID-19 patients were positively correlated with the level of IgM (r=0.255, P<0.05), while the levels of TNF-α were negatively correlated with the level of IgG (r=-0.217, P<0.05), but there were no significant correlation between the levels of IL-10, IL-1β, IL-6, MCP-1 and IL-4 and the concentrations of IgG and IgM.

2.5 Ήανγες οφ λεελ οφ ΙΛ-10, ΙΛ-1β, ΙΛ-6, ΜΆΠ-1, ΤΝΦ-α, ΙΠ-10 ανδ ΙΛ-4 ιν σερυμ σαμπλες οφ ΆΟΎΙΔ-19 πατιεντς

Except 2 dead patients, 17 asymptomatic infected patients and 3 patients with unknown condition, as Figure 2 shown, the other 26 COVID-19 patients need 13-42 days from onset to discharge, with an average of 26.3 days. The samples of the 26 COVID-19 patients were divided according to the interval time from onset to collection (random collection), and their course of disease -cytokine levels is shown in Figure 5. The changes of IL-1β and IL-6 were almost synchronous, increased sharply in 0-5 days and decreased sharply in 5-20 days. The changes of IL-10 and TNF-α in COVID-19 patients were almost synchronous, and the changes of MCP-1 and IL-4 were almost synchronous, they four all increased sharply in 20-25 days and decreased sharply in 25-30 days. IP-10 increased sharply in about 30 days after the onset of the disease, and then decreased sharply.

3 Discussion

A large number of Pro-inflammatory cytokines may lead to abnormal immune response when the immune system is unbalanced, and it may lead to systemic inflammatory response syndrome (SIRS)– cytokine release syndrome (CRS), also known as cytokine storm [6]. Cytokine storm can cause headache and fever, and it can cause disseminated intravascular coagulation and multiple organ system failure in severe cases [7]. SARS-CoV-2 is composed of RNA and nucleocapsid, which is covered with S protein[8], S protein of can be recognized by toll like receptor (TLR) [9], Angiotensin-Converting Enzyme 2 (ACE2)is the receptor for S protein to enter the cell[10,11], the ribosome of the infected cell will synthesize new infectious virus particles to infect other cells after S protein enters the cell [12,13], at this time, TLR and RIG I like receptor (RLR)can recognize the RNA of virus to activate the signal molecule TRIF; in addition, when stimulated by SARS-CoV-2, protein kinase C is activated by Ca^{2+} and translocating from cytoplasm to cell membrane, thus activating MAPK and NF-KB signaling pathways,the second one can recruit inflammatory nuclear factors to promote the gene expression of cytokines such as IL, IFN and TNF. The early symptoms of COVID-19 patients were atypical[14], with fever, dry cough and fatigue as the main manifestations, but severe patients may develop acute respiratory distress syndrome (ARDS), septic shock, metabolic acidosis that is difficult to treat, coagulation dysfunction or even multiple organ failure [4], which may be related to the levels of cytokines [15,16]. In addition, overactivation of T cells was found in COVID-19 patients [17]. The production of cytokines is related to the individual immune function and level, so the severity of the disease can be predicted according to its level. IL-1β is main produced by monocytes (MO), macrophages (Mφ), dendritic cells (DC), natural killer cells (NK), B-lymphocytes (B), it can stimulate the activation of T-lymphocytes (T cell), promote the proliferation and maturation of B cells and enhance the toxic and side effects of NK

cells by enhancing the expression of other Pro-inflammatory factors, it usually rises when acute rejection of transplantation (ART). The receptors of IL-6 and IL-4 are thrombopoietin receptor family; the former is produced by Th2, Mo, Mφ and DC, which can promote T cell proliferation and differentiation to helper T cell (Th) 1, and induce protein expression in acute phase; the latter is produced by Th2, basophil and mast cell, which can induce T cell differentiation to Th2, stimulate thymocyte and mast cell proliferation, and restrict Anti-inflammatory mediators; both of they can promote the proliferation and differentiation of B cells, produce antibodies, and they can promote the production of IgE synergistically. IL-10 is produced by Th2, Mo and Mφ, which can inhibit the secretion of Pro-inflammatory cytokines such as IFN-γ, down regulate the MHC II molecules of Mo, Mφ and DC, and inhibit the proliferation and differentiation of Th1; it can restrain anti-inflammatory mediators, control inflammatory reaction, and avoid excessive tissue damage, but it can promote the differentiation of B cells. The receptor of TNF-α is tumor necrosis factor receptor superfamily (TNFRSF), which is produced by Mo and Mφ· TNF-α in low concentration can stimulate Mo and Mφ to secrete IL-1 β, IL-6 and itself, and has tumor cytotoxic effect and antiviral effect to some extent; while it in high concentration has synergistic effect on IL-1β and IL-6, and can lead to tissue damage, disseminated intravascular coagulation and even septic shock; it is often increased in autoimmune disease (AID) and acute graft rejection (ART). Receptors of MCP-1 and IP-10 are chemokine receptor families (CHKR), and can chemotactic and activate Mo and T cells; the former is main produced by Mo, Mφ, B cells, and can activate basophils and DC, which play an important role in the occurrence and development of rheumatoid arthritis and other diseases; the latter is produced by T cells induced by interferon, which can activate and promote NK cell-mediated cytolysis, it is involved in the regulation of tumor angiogenesis.These cytokines interact with each other to promote the process of inflammation.

In order to determine the application value of the above-mentioned cytokine detection in the COVID-19 patients, the authors measured the level of these cytokine in 120 serum samples of 48 COVID-19 patients, 53 serum samples of patients with other diseases causing the rise of inflammatory factors and 35 serum samples of healthy people by ELISA, then the clinical characteristics and the expression of cytokines of patients with COVID-19 were analyzed combined with the detection results and relevant case data. The results showed that the level of IL-10, IL-1β, IL-6, MCP-1, TNF-αand IL-4 in the serum of COVID-19 patients were significantly higher than that in the serum of healthy people, while the serum levels of IP-10 between COVID-19 patients and healthy people were not significant different; the serum levels of IL-10, IL-1β, MCP-1, TNF-α, IP-10 and IL-4 in COVID-19 patients were significantly higher than those in other diseases patients, while the serum levels of IL-6 between COVID-19 patients and other diseases patients were not significant different. As of now, three highly pathogenic coronavirus diseases have been discovered, the previous two being Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS). In the serum samples of patients infected with those diseases, the levels of monocytes, macrophages, neutrophils and serum Pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, IP-10 and MCP-1 were always raised[18], while the level of IL-10 may be reduced[19].In contrast, the levels of IL-10 in the serum samples of COVID-19 patients were raised. It should be noted that the serum levels of IL-10, MCP-1, TNF-α and IL-4 in the male COVID-19 patients were significantly higher than those in the female patients; the serum levels of MCP-1 in the elderly were significantly higher than those in the youth and middle-aged patients; the serum levels of TNF-α and IL-4 in the elderly were significantly higher than those in the middle-aged patients. The serum levels of IP-10 in patients were positively correlated with IgM, while TNF-α were negatively correlated with IgG.

In summary, this study reveals that IL-10, IL-1β, IL-6, MCP-1, TNF-α and IL-4 are important markers for the detection of COVID-19.The combined diagnosis of IL-4 and TNF-α was seen as the most potent strategy, and its sensitivity (parallel experiment) and specificity (series experiment) reaching 99.8% and 99.9% respectively. The serum levels of IL-10, MCP-1, TNF-α and IL-4 in male COVID-19 patients were significantly higher than those in female patients, which may be related to the high levels of ACE2 in testes, seminiferous tubules and stromal cells [20,21], that cause the attack of SARS-CoV-2 on male reproductive system. The continuous monitoring of the cured COVID-19 patients showed that the levels of IL-1β and IL-6 in COVID-19 patients increased sharply in the early stage (0-5 days), and decreased gradually in

5-20 days, IL-6 can stimulate the promoter of SAA and CRP, and IL-1β has a synergistic effect with IL-6, the rise of SAA and CRP in the early stage of COVID-19 patients may be related to this [22,23]; IL-10, MCP-1, TNF-α and IL-4 rose sharply from 20 to 25 days, and declined sharply from 25 to 30 days; IP-10 rose sharply from 30 days after onset, and then declined sharply, which may because the immune system secreted more IP-10 to induce excessive apoptosis of immune cells in order to avoid losing its normal tissues in the late stage of inflammation, and the levels of IL-10, MCP-1, TNF-α and IL-4 also decreased. Due to the limited number of samples and enrollment, only 2 cases in this paper are dead patients, and there is no comparison of the levels of cytokines between light and severe patients, and only the cytokines in patients' serum are detected, and there is no discussion about the level of cytokines in patients' plasma and other body fluids. It has been reported that CRS often erupts in severe COVID-19 patients, and there are high levels of Pro-inflammatory cytokines such as IL-1β, IL-6, IL-7, IL-10, G-SCF, IP-10, MCP-1, TNF-α and IFN-γ in serum[1,24,25], to make it clear that whether the cytokine level of patients in the early stage of disease (before severe disease) can predict the severity of the course of disease development of COVID-19 patients, also needs more patients in the group and samples collected at a specific time for discussion and demonstration. The follow-up research will continue to increase the sample size to make the results more convincing.

Table 1.Serum levels of IL-10, IL-1β, IL-6, MCP-1, TNF-α, IP-10 and IL-4 of COVID-19 patients in different genders

?x±SD (pg/mL)

Group	n	?x±SD (pg/mL)	?x±SD (pg/mL)	?x±SD (pg/mL)	?x±SD (pg/mL)	?x±SD (pg/mL)	?x±SD (pg/m
		IL-10	IL-1β	IL-6	MCP-1	TNF-α	IP-10
male	75	9.39±2.11	6.30±0.61	12.95±4.25	129.02±24.63	22.70±3.60	92.23±24.46
female	37	5.29±0.65	7.73±0.81	8.69±1.19	69.62±13.74	18.50±2.25	53.42±20.29
P	/	0.017	0.057	0.278	0.004	0.035	0.085

Table 2.Serum levels of IL-10, IL-1β, IL-6, MCP-1, TNF-α, IP-10 and IL-4 in COVID-19 patients in different ages

?x±SD (pg/mL)

Group	n	?x±SD (pg/mL)	?x±SD (pg/mL)	?x±SD (pg/mL)	?x±SD (pg/mL)	?x±SD (pg/mL)	?x±SD
		IL-10	IL-1β	IL-6	MCP-1	TNF-α	IP-10
Young	46	6.20±2.55	6.10±0.78	5.93±0.78	71.82±28.74#	19.73±6.61	52.69±2
Middle-aged	32	6.71±1.28	6.55±0.87	10.87±1.85	85.11±17.18#	18.38±3.62#	79.44±3
Elderly	42	9.62±2.50	6.38±0.88	15.87±7.37	157.75±30.04	26.05±5.31	93.61±2
P value	/	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05

[a] #means P<0.05 when compared with elderly patients

Table 3.Correlation Analysis of serum levels of IL-10, IL-1β, IL-6, MCP-1, TNF-α, IP-10 and antibody in COVID-19 patients

Antibody	n	r	r	r	r	r	r	r
		IL-10	IL-1β	IL-6	MCP-1	TNF-α	IP-10	IL-4
IgM	120	0.078	-0.087	0.115	0.133	0.010	0.255*	-0.104
IgG	120	-0.150	-0.179	-0.103	-0.160	-0.217*	-0.097	-0.176
P	/	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05

[a]*means P<0.05。

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Acknowledgement

Fund project: Innovative provincial construction special novel coronavirus pneumonia emergency response special topic in Hunan province science and Technology Department 2020 (2020SK3009);Novel coronavirus molecular diagnosis technology and development and clinical application of mobile system(2020SK3018).

Conflict of Interest: The authors declare no conflicts of interest.