Int J Clin Exp Med 2020;13(11):8982-8989
www.ijcem.com /ISSN:1940-5901/IJCEM0119247

Original Article

Serum heme-oxygenase 1 and Toll-like receptor 4 expression in ischemic cerebrovascular disease and their relationship with disease prognosis

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Received July 30, 2020; Accepted August 26, 2020; Epub November 15, 2020; Published November 30, 2020

Abstract: Objective: Our aim was to explore the expression of serum heme oxygenase-1 (HO-1) and Toll-like receptor 4 (TLR4) in ischemic cerebrovascular disease (ICD) and their relationship with disease prognosis. Methods: We selected 311 ICD patients for the ICD group, and 311 subjects without cerebrovascular disease in the non-ICD group from the same study period. The expression levels of interleukin-6, C-reactive protein, tumor necrosis factor-α and HO-1 in serum and TLR4 in peripheral blood were compared between the two groups. In the ICD group, the patients were further divided into mild, moderate and severe stenosis groups according to the degree of cerebral artery stenosis, and into mild, moderate and severe injury groups according to the National Institutes of Health Stroke Scale (NIHSS) score. The expression levels of the above indicators were compared among the three subgroups. Besides, the correlation between the indicator levels and the degree of stenosis and NIHSS score in the ICD group were analyzed. After 3 months of follow-up, the ICD group was further classified as a good prognosis group and a poor prognosis group according to the modified Rankin Scale score, and the indicator levels were also compared between the two subgroups. Then the clinical value of HO-1 and TLR4 levels in assessing the prognosis of ICD was analyzed. Results: The expression levels of interleukin-6, C-reactive protein, tumor necrosis factor-α, HO-1 and TLR4 in the ICD group were significantly improved (all $P<0.05$), as compared with those in the non-ICD group. As the degree of stenosis or injury became more severe, the indicator levels in the ICD group increased accordingly, which were positively correlated with the degree of stenosis and NIHSS score (all $P<0.05$). Moreover, the poor prognosis group showed much higher indicator levels than the good prognosis group (all $P<0.05$), and receiver operating characteristic results demonstrated that the area under the curve of HO-1 and TLR4 in evaluating the prognosis of patients were >0.800. Conclusion: The expression levels of HO-1 and TLR4 in ICD patients are significantly increased and positively correlated with the severity of the disease, which has a certain clinical value in evaluating the prognosis of ICD patients.

Keywords: Ischemic cerebrovascular disease, heme oxygenase-1, Toll-like receptor 4, prognosis

Introduction

Ischemic cerebrovascular disease (ICD) is characterized by brain tissue necrosis or loss of cerebral function resulting from insufficient blood supply. Due to ischemia and hypoxia, ICD patients may have different degrees of brain damage functionally, which exerts a negative impact on cognitive and neurological function, and poses a serious threat to the quality of life and health of patients [1-3]. Recent epidemiological data shows that the incidence of ICD accounts for 65%-85% of cerebrovascular diseases and its incidence is still increasing with a poor prognoses in some patients [4, 5]. Therefore, it is of great clinical significance to explore specific indicators of prognosis for ICD patients.

ICD is usually a consequence of atherosclerosis, which is mainly caused by an inflammatory response and oxidative stress [6, 7]. Heme oxy-

Table 1. General data (n, $\bar{x} \pm sd$)

Category	ICD group (n=311)	Non-ICD group (n=311)	χ^2/t	P
Age (year)	49.7±5.4	50.1±4.9	0.967	0.334
BMI (kg/m^2)	24.4±2.3	24.5±2.6	0.508	0.612
Smoking history			63.605	<0.001
Yes	157	62		
No	154	249		
Drinking history			34.446	<0.001
Yes	132	64		
No	179	247		
Hypertension history			26.602	<0.001
Yes	76	28		
No	235	283		
Diabetes history			27.907	<0.001
Yes	73	25		
No	238	286		
History of coronary heart disease			33.336	<0.001
Yes	89	32		
No	222	279		
Hyperuricemia			32.718	<0.001
Yes	67	18		
No	244	293		
Atrial fibrillation			18.561	<0.001
Yes	44	13		
No	267	298		

genase 1 (HO-1), the rate-limiting enzyme in heme degradation, plays a role in anti-oxidation and anti-apoptosis *in vivo* [8-10]. Toll-like receptors (TLRs), pattern recognition receptors that recognize pathogen-associated molecular patterns, plays an important role in the innate immune system. What's more, TLRs are closely associated with the occurrence and development of atherosclerosis, and vitally involved in the formation of vulnerable plaques of atherosclerosis [11, 12]. Currently, there are few reports on HO-1 and TLR4 expression in evaluating the prognosis of ICD patients. Based on the research findings, our study investigated the expression levels of HO-1 and TLR4, and their correlation with the prognosis of ICD, so as to provide a relevant basis for the clinical treatment and prognostic evaluation.

Materials and methods

General data

We recruited 311 ICD patients admitted to the Affiliated Renhe Hospital of China, Three Gorges University, Second Clinical Medical College of China Three Gorges University from January 2016 to January 2019 into the ICD group, and 311 sex- and age-matched subjects without ICD in the non-ICD group during the same period. There were 183 males and 128 females with a mean age of 49.7±5.4 years in the ICD group, and 183 males and 128 females with a mean age of 50.1±4.9 years in the non-ICD group. No statistically significant difference was revealed in age and gender between the two groups (P>0.05). See **Table 1** for more details. Written informed consent was obtained from all patients and their families. Ethical approval for the study was given by the Ethics Committee of Affiliated Renhe Hospital of China Three Gorges University, Second Clinical Medical College of China Three Gorges University.

Inclusion and exclusion criteria

Patients in the ICD group were included if they were diagnosed with ICD according to the criteria issued by the fourth National Academic Conference on Cerebrovascular Disease [13]; had first onset with onset-to-admission time ≤24 h; were aged ≥18 years. Subjects in the non-ICD group were included if they had no cerebrovascular diseases according to the physical examination within the past month; were aged ≥18 years.

In addition, patients were excluded in both groups if they had malignant tumors; were complicated with heart disease that might lead to cerebral embolism, severe cognitive impairment, infectious diseases or inflammatory symptoms in the past month, or other severe diseases; were administered anti-inflammatory drugs, anahormones, immunosuppressive

Table 2. Primer sequences

Primer	Sequence
TLR4 (Upstream)	5'-TGGATACGTTTCTTATAA-3'
TLR4 (Downstream)	5'-GAAATGGAGGCACCCCTT-3'
β-actin (Upstream)	5'-GAGCCTCGCCTTTGCCGATCC-3'
β-actin (Downstream)	5'-CGATGCCGTCTCGATGGGG-3'

agents, etc. that might affect the experimental results in the past 3 months.

Methods

Fasting venous blood samples were drawn from each subject in the ICD group into two 3 mL tubes, the next morning after enrollment (within 24 hours before treatment). A tube of blood was centrifuged at 3,000 rpm for 5 min to separate the serum, followed by measurement of interleukin-6 (IL-6), C-reactive protein (CRP), tumor necrosis factor (TNF-α) and HO-1 levels using enzyme-linked immunosorbent assay (ELISA) (Hamilton Microlab STAR Multimode Plate Reader, Switzerland). The other sample of blood was used to extract RNA from peripheral blood using an RNA extraction kit. Subsequently, reverse transcription reaction and reverse transcription polymerase chain reaction (RT-PCR) were performed according to the instructions of reverse transcription kit (RR047A, Takara, Japan) and SYBR RT-PCR kit (BSB23, Hangzhou Bioer Technology Co., Ltd.), respectively. The reaction conditions were as follows: pre-denaturation at 95°C for 30 s, 35 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 30 s. Then the relative expression of TLR4 and β-actin genes in each group was determined by the $2^{-\Delta\Delta Ct}$ method, with β-actin as the internal reference. Each measurement was repeated three times and then the results were averaged. Primers were synthesized by Beijing Dingguo Changsheng Biotechnology Co., Ltd., and the sequences are shown in **Table 2**.

Outcome measures

The expression levels of IL-6, CRP, TNF-α, HO-1 in serum and TLR4 mRNA in peripheral blood were compared between the two groups.

In the ICD group, the degree of vascular stenosis was graded using the North American Symptomatic Carotid Endarterectomy Trial (NASCET), as mild stenosis (10%-29%), moderate stenosis (30%-69%), and severe stenosis (>70%) [14]. Correspondingly, those with ICD were divided into mild, moderate and severe stenosis groups. The expression levels of IL-6, CRP, TNF-α and HO-1 in serum and TLR4 mRNA in the peripheral blood at different grades were compared.

Additionally, the ICD group was assessed by the National Institutes of Health Stroke Scale (NIHSS) at enrollment and divided into three subgroups, namely, the mild injury group (NIHSS score ≤4), the moderate injury group (NIHSS score 4-20); and the severe injury group (NIHSS score >20) [15]. The expression levels of the above indicators were compared between the three different subgroups.

After 3 months, a follow-up of patients from the ICD group was performed and the prognosis was evaluated by the modified Rankin Scale (mRS). Those with ICD were classified into good prognosis group (mRS 0-2) and poor prognosis group (mRS >2) [16]. The indicator levels were also compared between the two subgroups.

Pearson and Spearman correlation coefficients were used to analyze the correlation between the expression levels of IL-6, CRP, TNF-α and HO-1 in serum and TLR4 mRNA in the peripheral blood and NIHSS score and the degree of stenosis in the ICD group.

Receiver operating characteristic (ROC) curve analysis was applied to assess the clinical value of HO-1 and TLR4 in the diagnosis of ICD.

Statistical analysis

Data analyses were performed with the SPSS 22.0 software. Chi-square test (χ^2 test) was adopted for the comparison of enumeration data expressed as the percentage/case (n/%). The measurement data were expressed as mean ± standard deviation ($\bar{x} \pm sd$). Analysis of variance was used for the multigroup comparison, and LSD-t test was adopted for the intergroup comparison if the data were in accord with homogeneity of variance; if not, Dunnett's T3 test was used. Besides, Pearson or Spearman methods were applied to analyze the correlations. $P<0.05$ was considered statistically different. ROC curve analysis was used

Table 3. Comparison of relevant indicators between the two groups ($\bar{x}$ ± sd)

Group	IL-6 (pg/mL)	TNF-α (pg/mL)	CRP (mg/L)	TLR4 mRNA	HO-1 (ng/mL)
Non-ICD group (n=311)	49.82±5.59	2.98±1.02	1.19±0.46	0.23±0.11	2.01±1.12
ICD group (n=311)	187.32±21.47	13.14±3.36	8.92±2.23	1.10±0.54	4.15±2.53
T	109.297	51.026	59.870	27.841	13.640
P	<0.001	<0.001	<0.001	<0.001	<0.001

Note: IL-6: interleukin-6; CRP: C-reactive protein; TLR: Toll-like receptor; TNF-α: tumor necrosis factor; HO-1: Heme-oxygenase 1; ICD: ischemic cerebrovascular disease.

Table 4. Comparison of indicators at different grades of stenosis ($\bar{x}$ ± sd)

Group	IL-6 (pg/mL)	TNF-α (pg/mL)	CRP (mg/L)	TLR4 mRNA	HO-1 (ng/mL)
Mild stenosis group (n=89)	132.17±21.38	10.96±1.21	6.87±1.94	0.85±0.42	3.19±1.82
Moderate stenosis group (n=147)	193.46±31.24*	13.44±3.17*	9.02±2.36*	1.09±0.51*	4.17±2.36*
Severe stenosis group (n=75)	248.72±28.66*,#	17.25±2.23*,#	11.33±2.15*,#	1.36±0.45*,#	5.82±2.25*,#
F	352.803	127.224	83.977	23.814	29.709
P	<0.001	<0.001	<0.001	<0.001	<0.001

Note: Compared with the mild stenosis group, *P<0.05; compared with the moderate stenosis group, #P<0.05. IL-6: interleukin-6; CRP: C-reactive protein; TLR: Toll-like receptor; TNF-α: tumor necrosis factor; HO-1: Heme-oxygenase 1.

Table 5. Comparison of indicators at different grades of injury ($\bar{x}$ ± sd)

Group	IL-6 (pg/mL)	TNF-α (pg/mL)	CRP (mg/L)	TLR4 mRNA	HO-1 (ng/mL)
Mild injury group (n=84)	136.22±24.31	10.54±1.09	6.92±2.01	0.81±0.39	3.03±1.95
Moderate injury group (n=149)	192.56±30.29a	13.81±3.28a	9.14±2.12a	1.14±0.61a	4.45±2.29a
Severe injury group (n=78)	240.58±28.13a,b	16.25±2.17a,b	11.02±2.44a,b	1.54±0.38a,b	5.11±2.34a,b
F	277.813	100.472	72.261	42.184	14.096
P	<0.001	<0.001	<0.001	<0.001	<0.001

Note: Compared with the mild injury group, aP<0.05; compared with the moderate injury group, bP<0.05. IL-6: interleukin-6; CRP: C-reactive protein; TLR: Toll-like receptor; TNF-α: tumor necrosis factor; HO-1: Heme-oxygenase 1.

to assess the clinical value of HO-1 and TLR4 in diagnosing ICD.

Results

Comparison of relevant indicators between the two groups

The expression levels of IL-6, CRP, TNF-α and HO-1 in serum and TLR4 mRNA in peripheral blood were significantly higher in the ICD group than in the non-ICD group (P<0.05). See **Table 3**.

Comparison of indicators at different grades of stenosis

The expression levels of IL-6, CRP, TNF-α and HO-1 in serum and TLR4 mRNA in peripheral blood were higher in the moderate and severe stenosis group than in the mild stenosis group (all P<0.05), and markedly higher in the severe stenosis group than in the moderate stenosis group (all P<0.05). See **Table 4**.

Comparison of indicators at different grades of injury

The expression levels of IL-6, CRP, TNF-α and HO-1 in serum and TLR4 mRNA in peripheral blood were higher in the moderate and severe injury group than in the mild injury group (all P<0.05), and significantly higher in the severe injury group than in the moderate injury group (all P<0.05). See **Table 5**.

Correlation analysis results

The expression levels of IL-6, CRP, TNF-α and HO-1 in serum and TLR4 mRNA in peripheral blood in the ICD group were positively correlat-

Table 6. Correlation analysis results

Indicators	IL-6 (pg/mL)	TNF-α (pg/mL)	CRP (mg/L)	TLR4 mRNA	HO-1 (ng/mL)
Degree of stenosis					
Rs	0.483	0.734	0.556	0.864	0.761
P	0.004	0.021	<0.001	<0.001	<0.001
NIHSS					
R	0.552	0.671	0.484	0.842	0.788
P	<0.001	<0.001	0.017	<0.001	<0.001

Note: IL-6: interleukin-6; CRP: C-reactive protein; TLR: Toll-like receptor; TNF-α: tumor necrosis factor; HO-1: Heme-oxygenase 1; NIHSS: National Institutes of Health Stroke Scale.

Table 7. Comparison of indicators of different prognosis ($\bar{x} \pm sd$)

Group	IL-6 (pg/mL)	TNF-α (pg/mL)	CRP (mg/L)	TLR4 mRNA	HO-1 (ng/mL)
Good prognosis group (n=229)	168.65±28.73	11.53±2.12	7.88±2.42	0.86±0.47	3.37±1.36
Poor prognosis group (n=82)	250.48±29.16	18.01±2.23	11.97±2.51	1.77±0.65	6.32±2.49
T	22.045	23.426	13.004	11.635	10.197
P	<0.001	<0.001	<0.001	<0.001	<0.001

Note: IL-6: interleukin-6; CRP: C-reactive protein; TLR: Toll-like receptor; TNF-α: tumor necrosis factor; HO-1: Heme-oxygenase 1.

Table 8. ROC curve results for relevant indicators

Indicators	Cut off value	AUC	P	95% CI	Sensitivity	Specificity
TLR4	1.229	0.834	<0.001	0.784, 0.884	0.805	0.808
HO-1 (ng/mL)	3.595	0.818	<0.001	0.767, 0.869	0.854	0.734

Note: TLR: Toll-like receptor; HO-1: Heme-oxygenase 1; AUC: area under curve; CI: confidence interval; ROC: receiver operating characteristic.

ed with the degree of stenosis and NIHSS score (all P<0.05). See **Table 6**.

Comparison of indicators of different prognosis

The poor prognosis group showed higher expression levels of IL-6, CRP, TNF-α and HO-1 in serum and TLR4 mRNA in peripheral blood than the good prognosis group. See **Table 7**.

ROC curve results for relevant indicators

According to ROC curve analysis, if the TLR4 cutoff value was 1.229 or the HO-1 cutoff value was 3.595 ng/mL, the area under curve was >0.800 in evaluating the prognosis of patients. See **Table 8** and **Figure 1**.

Discussion

Inflammation plays a significant role in the occurrence and development of atherosclerosis. CRP is a non-specific marker of inflammation in the body, and CRP levels increase significantly when infectious diseases or inflammatory responses occur. IL-6 can lead to tissue damage through cellular and humoral immunity, promoting the formation of atherosclerosis. TNF-α is overexpressed in atherosclerosis with a wide range of biological characteristics, which can facilitate the release of oxygen free radicals and aggravate injuries [17]. In our study, the expression levels of IL-6, CRP and TNF-α in ICD patients were significantly increased as compared with those of the population controls, and were positively correlated with the severity of the disease. The results suggest that inflammatory factors are closely associated with the occurrence and development of ICD.

ICD is mainly caused by cerebral artery thrombosis and its pathogenesis is still unclear. It was reported that oxidative reactions play a critical role in the occurrence and development of ICD [18]. HO-1 as a key intracellular antioxidant enzyme can produce biliverdin, carbon monoxide (CO) and iron ions by degrading

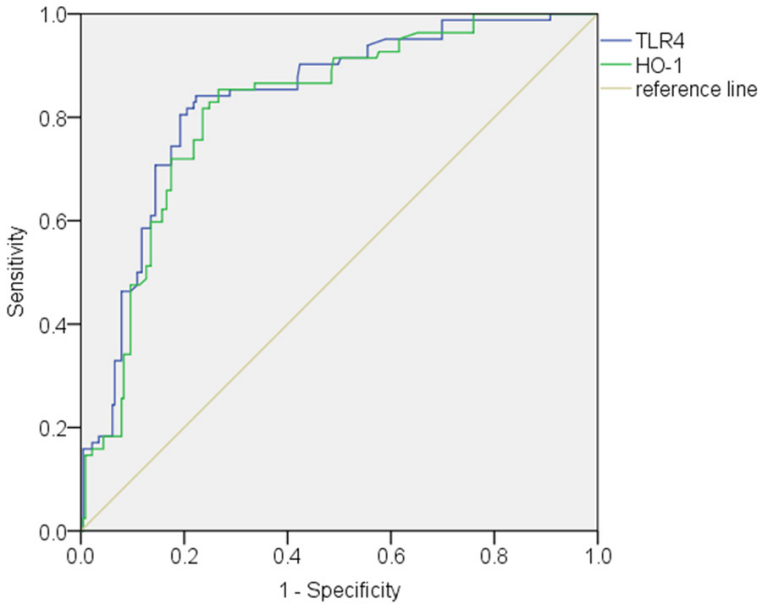

Figure 1. ROC for TLR4 and HO-1 expression levels in evaluating the prognosis of ICD. TLR: Toll-like receptor; HO-1: Heme-oxygenase 1; ICD: ischemic cerebrovascular disease; ROC: receiver operating characteristic.

heme, and bilirubin is then formed by the action of biliverdin reductase. A previous study revealed that endogenous protective systems composed of HO-1, bilirubin, and CO were important in anti-oxidative stress and anti-inflammatory responses [19]. Bilirubin and biliverdin can inhibit lipid peroxidation, thereby removing excessive reactive oxygen species from the body, while CO can suppress endothelial cell apoptosis and promote endothelial cell repair and proliferation around the injured area, thereby reducing oxidative stress injury. An *in vivo* study showed that atherosclerosis in animals could be inhibited by upregulating the HO-1 pathway, and aggravated by downregulating the HO-1 pathway [20]. Furthermore, HO-1 was found to be involved in inflammation-related brain injury protection [21]. In our study, we identified that serum HO-1 levels were significantly increased in ICD patients, suggesting that the expression levels of serum HO-1 as a protective role in ICD, which was consistent with the results of similar studies. TLRs, type I transmembrane receptors, are widely distributed in endothelial cells, epithelial cells, dendritic cells and macrophages, considered as the only class of transmembrane proteins in mammals that can transmit extracellular information to cells and induce inflammatory responses. TLR4, is the first discovered and most widely studied class of receptors in the TLR family, it can bind to a variety of exogenous or endogenous ligands to exert biological effects. Inflammation is one of the main risk factors for the development of atherosclerosis. Studies have unveiled that TLRs can promote the expression of inflammatory factors, such as IL-6 and TNF-α, thus mediating vascular endothelial inflammatory injury [22, 23].

Besides, our study revealed that the TLR4 mRNA levels of ICD patients were significantly increased as compared with those of normal controls, and TLR4 and HO-1 levels were positively correlated with the degree of stenosis and injury in patients (more severe stenosis or neurological injury reveals higher levels of TLR4 and HO-1). The results suggest that TLR4 and HO-1 may be involved in the occurrence and development of ICD. We further explored the clinical value of TLR4 and HO-1 for prognosis of ICD patients, and the results demonstrate that both TLR4 and HO-1 can act as good auxiliary indicators to evaluate the prognosis of ICD patients.

However, there are still some shortcomings in this study. We did not dynamically observe the changes of each indicator, nor investigat the correlations between indicator levels and prognosis at different time points; hence, further studies are still needed to get a more precise conclusions in the future.

To sum up, the expression levels of HO-1 and TLR4 are significantly increased in ICD and positively correlated with the severity of the disease, which are of certain clinical value in evaluating the prognosis of ICD patients.

Disclosure of conflict of interest

None.

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