High serum OX40 ligand correlates with severity and mortality in patients with massive cerebral infarction

Lun-Lin Mao, MMa,b, Wen-Ya Chen, BMa,b,∗, Ai-Jin Ma, BMa,b, Li-Li Ji, MMa,b, Ting-Ting Huang, MMa,b

Abstract
OX40 ligand (OX40L) is a member of tumor necrosis factors (TNF)/TNFR superfamily and is mainly expressed in activated T cells and participates in various inflammatory reactions. However, it remains unclear about the role of serum OX40L as a biomarker of cerebral infarction (CI). This study aimed to explore the possibility of serum OX40L as a meaningful predictor in mortality of CI. Severe CI patients were included to conduct clinicopathological and laboratory data and measure serum OX40L level. Patients were followed up after discharge and 60-day survival rate was used as the study endpoint. The results showed that of all 294 patients, 123 (41.8%) died within 60 days after admission. Serum OX40L levels were significantly higher in patients with severe CI compared to healthy controls, and were significantly higher in nonsurvivors compared to survivors (P < .05). The levels of OX40L were correlated with Glasgow Coma Scale score, serum creatinine and high-sensitive C-reactive protein. Multivariate logistic regression analysis showed that serum OX40L level was an independent prognostic factor for 60-day mortality, after control of pulmonary infection, Glasgow coma scale score and high-sensitive C-reactive protein (odds ratio = 1.089; 95% confidence interval = 1.053–1.126; P < .001). The receiver operating characteristic (ROC) curve was used to predict the best cut-off of serum OX40L for 60-day survival as 35.5 ng/mL. Patients with high serum OX40L levels (>35.5 ng/mL) had a significantly higher mortality within 60 days (hazard ratio = 2.885; 95% confidence interval = 1.901–4.378). In conclusion, OX40L is a serum biomarker of patients with CI and associated with severity and mortality of this disease.

Abbreviations: ACI = atherosclerotic cerebral infarction, CI = cerebral infarction, hs-CRP = high-sensitive C-reactive protein, OX40L = OX40 ligand, TNF = tumor necrosis factors, WBC = white blood cells.

Keywords: biomarker, cerebral infarction, ischemic stroke, OX40 ligand

1. Introduction
Cerebral infarction (CI) is 1 common ischemic stroke associated with severe health burden, disability and mortality.[1] In CI, blood clots cause cerebral blood flow disruption and blood vessel obstruction, resulting in local brain tissue ischemia and cell death. In addition, CI is often accompanied by secondary lesions such as inflammation, apoptosis, oxidative stress, increased vascular permeability, and cerebral edema, which further promote neural cell death.[2] A key pathophysiological process of ischemic stroke is inflammatory response, especially in the acute phase after the onset of CI, accompanied by higher concentration of proinflammatory cytokines, such as C-reactive protein (CRP).[3,4] Then the blood-brain barrier is damaged, which in turn activates peripheral immune cells such as neutrophils and T cells to infiltrate and accumulate in ischemic brain area.[5] Inflammation may play a dual role in ischemic brain tissue. It may promote inflammation of the brain through inflammatory mediators and increase tissue damage. It may also facilitate the repair process by accelerating the removal of necrotic debris.[6] Increasing evidence has confirmed that smaller infarct size and better clinical outcomes can be achieved by reducing pro-inflammatory cytokines and increasing anti-inflammatory cytokines, and suggests that the prognosis is determined by the balance between inflammatory and anti-inflammatory cytokines.[7] Therefore, biomarkers of proinflammatory or anti-inflammatory cytokines may be an early diagnostic and prognostic indicator of ischemic stroke.

OX40 ligand (OX40L, CD252) and its receptor OX40 (CD134) are members of TNF superfamily.[8] OX40L is mainly expressed by immune cells, such as B cells, macrophages and dendritic cells.[9] Rosuvastatin is an effective drug for controlling atherosclerosis. Recent studies have reported that rosuvastatin significantly reduced OX40L expression in peripheral blood lymphocyte and plasma soluble OX40L in patients with atherosclerotic CI (ACI).[10] This suggests that OX40/OX40L may promote atherosclerosis in ACI.
patients. However, the clinical significance of serum OX40L in patients with CI is unclear.

In this study, we measured serum soluble OX40L concentration and analyzed its relationship with clinical severity and mortality in patients with CI. Our study will identify OX40L as a serum biomarker for predicting prognosis in patients with CI.

2. Materials and methods

2.1. Subjects

A prospective study was conducted in 294 patients with acute CI. They were admitted to the Department of Neurology in Wujin Hospital Affiliated to Jiangsu University, from January 2014 to December 2017. The diagnosis of massive CI was confirmed by computed tomography or magnetic resonance imaging. The severity of CI was evaluated according to the Glasgow Coma Scale (GCS), and the severe CI was defined as when GCS<9. Exclusion criteria included previous ischemic stroke, intracerebral hemorrhage or subarachnoid hemorrhage, brain tumor, infectious brain disease, and previous brain trauma. The clinical data, medical history and laboratory indicators were recorded for each patient. Serum of 120 gender and age-matched healthy donors were selected as controls. All patients were regularly followed-up for at least 60 months. The study was approved by the ethics committee of Wujin Hospital Affiliated to Jiangsu University and conducted in accordance with the Declaration of Helsinki. The patient or family member signed the informed consent.

2.2. Measurement of clinical and laboratory variables

We recorded the following clinical variables for each patient: gender, age, atrial fibrillation, hypertension, pulmonary infection, cardiac insufficiency, diabetes, and hyperlipidemia. The acute physiology and immunological chronic health assessment II score was used to estimate systemic organ damage. The patient’s laboratory variables were measured: white blood cells, platelets, lactic acid, glucose, creatinine, and high-sensitive C-reactive protein (hs-CRP). The end point study was a 60-day mortality rate.

2.3. ELISA detection of serum OX40L

Blood samples were collected within 24 hours of hospital admission to separate serum, and stored at -80°C. Serum OX40L levels were measured by ELISA using Human Soluble OX40L kit (Abebio, cat. no. AE13973HU), and absorbance at 450nm was measured with a microplate reader. OX40L concentrations were calculated according to the standard curve established by different concentrations of recombinant human OX40L, and expressed as pg/mL for each case.

2.4. Statistical analysis

SPSS19.0 statistical software was used to analyze the data. Continuous variables were presented as median (interquartile range) and analyzed using the Wilcoxon-Mann-Whitney test. Categorical variables were presented as frequency (percentage) and analyzed using the chi-square test. The correlation between OX40L and continuous variables was analyzed using the Spearman’s rank correlation test. Multivariate logistic regression analysis determine the independent contribution of OX40L on mortality, and expressed as odds ratio and the 95% confidence interval. The receiver operating characteristic curve was plotted to determine the cut-off point of serum OX40L between the survivors and the nonsurvivors. Kaplan-Meier curves were used to analyze the effect of high serum OX40L levels (≥35.5 pg/mL) on 60-day mortality in patients with CI. P < .05 was considered as a significant criterion for the difference.

3. Results

3.1. Baseline characteristics of study samples

Of the 294 patients with CI, the median age was 79 years old, with 121 males and 173 females. The clinical pathological

Table 1

| Characteristics         | Cerebral infarction n = 294 | Survivors n = 171 | Nonsurvivors n = 123 | P value |
|-------------------------|-----------------------------|------------------|---------------------|---------|
| Male sex†               | 121 (41.2%)                 | 72 (42.1%)       | 49 (39.8%)          | .697    |
| Age (yr)†               | 79 (73–84)                  | 79 (72–84)       | 80 (73–85)          | .576    |
| Atrial fibrillation†    | 213 (72.4%)                 | 118 (69.0%)      | 95 (77.2%)          | .119    |
| Hypertension†           | 219 (74.5%)                 | 124 (72.5%)      | 95 (77.2%)          | .360    |
| Pulmonary infection†    | 152 (51.7%)                 | 77 (45.0%)       | 75 (61.0%)          | .007    |
| Cardiac insufficiency†  | 105 (35.7%)                 | 54 (31.6%)       | 51 (41.5%)          | .081    |
| Diabetes mellitus†      | 44 (15.0%)                  | 29 (17.0%)       | 15 (12.2%)          | .259    |
| Hyperlipidemia†         | 31 (10.5%)                  | 17 (9.9%)        | 14 (11.4%)          | .692    |
| GCS score               | 6 (5–8)                     | 7 (6–8)          | 6 (4–7)             | <.001   |
| APACHE II score*        | 22 (19–23)                  | 21 (19–23)       | 22 (19–24)          | .195    |
| WBC (10^3 cells/μL)*    | 7 (6.4–7.8)                 | 6.9 (6.3–7.8)    | 7.4 (6.4–8.3)       | .037    |
| Platelets (10^3 cells/μL)* | 147 (128–164)               | 148 (130–168)    | 143 (124–158)       | .010    |
| Lactic acid (mmol/L)    | 2.65 (2.33–3.1)             | 2.66 (2.36–3.09) | 2.64 (2.29–3.1)     | .751    |
| Glucose (mmol/L)        | 6.2 (5.4–7.1)               | 6.2 (5.5–7.1)    | 6.1 (5.1–7)         | .186    |
| Creatinine (μmol/L)*    | 92 (74–109)                 | 87 (69–107)      | 95 (77–111)         | .025    |
| hs-CRP (pg/mL)†         | 2.6 (2.2–2.9)               | 2.6 (2.2–2.8)    | 2.7 (2.3–3.1)       | <.001   |
| OX40L (ng/mL)†          | 37 (32–43)                  | 37 (29–41)       | 41 (36–46)          | <.001   |

APACHE = Acute Physiology and Chronic Health Evaluation, GSC = Glasgow Coma Scale, hs-CRP = high sensitive C-reactive protein, WBC = white blood cell count.
† Continuous variable are expressed as median (25th to 75th percentiles) and analyzed by Mann-Whitney U test.
† Categorical Variable are expressed as frequency (%) and analyzed by Chi-squared test.
features of CI admission are shown in Table 1. Within 60 days after onset, 123 patients died and the mortality rate was 41.8%. These nonsurvivors had significantly higher white blood cells, serum creatinine and hs-CRP levels, and significantly lower GCS scores and platelets compared to survivors (all \( P < .05 \)). At the same time, nonsurvivors had a higher frequency of pulmonary infection than survivors (all \( P < .05 \)).

### 3.2. Serum OX40L levels were elevated in CI patients

We measured serum OX40L concentrations by ELISA assay. Patients with CI (median 37 ng/mL) had significantly elevated serum OX40L levels than the control group (median 24.5 ng/mL) \( (P < .001) \) (Fig. 1A). In patients with CI, serum OX40L levels were significantly higher in nonsurvivors (median 41 ng/mL) than in survivors (median 35 ng/mL) \( (P < .001) \) (Fig. 1B).

### 3.3. Correlations of serum OX40L with clinical variables of CI

We performed Spearman rank correlation test to determine the associations of OX40L with clinical severity of CI. Serum OX40L correlated negatively to GCS score \( (r = 0.161, P = .006) \) (Fig. 2A), and correlated positively to serum creatinine \( (r = 0.198, P = .001) \) (Fig. 2B) and hs-CRP \( (r = 0.167, P = .004) \) (Fig. 2C).

### 3.4. Serum OX40L predicted 60-day mortality of CI

Multivariate logistic regression analysis demonstrated that serum OX40L level is an independent contributor of 60-day mortality after controlling for pulmonary infection, GCS score and hs-CRP (odds ratio = 1.089; 95% confidence interval = 1.053–1.126; \( P < .001 \)) (Table 2). Receiver operating characteristic (ROC) curve was plotted to determine the cut-off point of serum OX40L was 35.5 ng/mL (sensitivity = 76.4%, specificity = 56.1%), so as to differentiate survivors and nonsurvivors (Fig. 3A). It was found that serum OX40L levels predicted a 60-day mortality area (AUC) of 0.701% (95% confidence interval = 0.642–0.760; \( P < .001 \)). Using the Kaplan-Meier curve for survival analysis, patients with serum OX40L levels above 35.5 ng/mL showed a significantly higher 60-day mortality rate (hazard ratio = 2.885; 95% confidence interval = 1.901–4.378; Log Rank = 19.569; \( P < .001 \)) (Fig. 3B).
4. Discussion

This study investigated serum OX40L levels in 294 patients with CI. We found that serum OX40L levels were elevated in patients with CI compared to age- and sex-matched healthy controls, and were also higher in nonsurvivors than in survivors. Serum OX40L showed negative correlation with GCS score, and showed positive correlation with serum creatinine and hs-CRP in CI patients. Serum OX40L, as well as pulmonary infection, GCS score and hs-CRP, contributed independently to 60-day mortality of patients with CI. Serum OX40L, as well as pulmonary infection, GCS score and hs-CRP, contributed independently to 60-day mortality of patients with CI. Patients with higher serum OX40L levels (≧35.5ng/mL) have a higher mortality rate at 60 days, so OX40L may be a good serum biomarker in patients with CI.

This study is the first report about the elevation of serum OX40L in patients with CI. Previous studies have supported our results and showed OX40L in peripheral blood lymphocytes and plasma can be reduced by rosuvastatin and simvastatin in patients with ACI.[10,12] In fact, statins seem to has a common effect on OX40L, as pitavastatin or simvastatin could also repressed the expressions of OX40L Dendritic cells.[13] Statins control atherosclerosis by inhibiting the inflammatory response and release of inflammatory factors.[14] It can be speculated that OX40L may be an inflammatory factor induced by CI. However, this study reported a new finding about the correlation between high serum OX40L and mortality of patients with CI, which is consistent with previous studies. For example, serum OX40L was associated with the severity and persistence of asthma in children, and associated with the prognosis of patients with acute coronary syndrome.[15,16] After middle cerebral artery occlusion (MCAO), rats had significantly higher OX40 expression in the ischemic brain tissue and peripheral blood.[17] OX40 is a receptor for OX40L, suggesting that occlusion of the middle cerebral artery may activate the OX40L-OX40 signaling pathway. This article shows higher serum OX40L levels in patients with CI compared to the control group, thus confirming this hypothesis.

We reported that serum OX40L correlated negatively to GCS score, and correlated positively to serum creatinine and hs-CRP in CI patients. Serum OX40L showed negative correlation with GCS score, and showed positive correlation with serum creatinine and hs-CRP in CI patients. GCS score is a coma index and was routinely used to assess the conscious state of patients. It measures blink reaction, language reaction and limb movement, with lower GCS score indicating higher extent of unconsciousness and disease severity. This indicates that OX40L might be a contributor to the progression of CI. In our study, serum OX40L showed positive correlation with serum creatinine, an indicator of renal function and prognosis of ischemic stroke, which was also reported by other scholars in patients with systemic lupus erythematosus.[18,19] We also found that serum OX40L positively correlated with a proinflammatory cytokine hs-CRP. OX40L also demonstrated positive correlation with hs-CRP in acute coronary syndrome patients, and both these 2 proteins involve the pathogenesis of atherosclerosis, and associated with disease severity and progression.[20] Moreover, serum OX40L and hs-CRP can both be reduced by rosuvastatin treatment in acute ACI patients.[10] This suggests that there might be a regulatory connection between OX40L and hs-CRP, which needs further investigation.

Our study showed that patients with higher serum OX40L had worse clinical outcomes within 60 days, suggesting that OX40L may be an inflammatory factor and could predict the prognosis of CI. Therefore, we speculate that OX40L signaling might play an key role in pathophysiological mechanisms of CI. The association of high serum OX40L with poor prognosis may be ascribed to following pathways: First, regulatory T cells can inhibit the activation of immune responses during ischemic stroke and reduce inflammation-mediated brain injury.[21] For example, in rats with transient middle cerebral artery occlusion, atorvastatin

| Characteristics | Odds ratio | 95% confidence interval | P value |
|-----------------|------------|------------------------|---------|
| Pulmonary infection | 1.767 | 1.049–2.976 | .032 |
| GCS score | 0.791 | 0.681–0.919 | .002 |
| Hs-CRP (pg/mL) | 2.216 | 1.347–3.643 | .002 |
| OX40L (ng/mL) | 1.089 | 1.053–1.126 | <.001 |

GCS = Glasgow Coma Scale, hs-CRP = high sensitive C-reactive protein.

Figure 3. High serum OX40L is associated with high mortality in patients with cerebral infarction. (A) The ROC curve was used to determine the cut-off point for serum OX40L that distinguishes the survivors and nonsurvivors. (B) Kaplan-Meier survival curves show significantly higher 60-d mortality in patients with high serum OX40L (≧35.5ng/mL) compared to patients with low serum OX40L (<35.5ng/mL). OX40L = OX40 ligand.
increases the number and proportion of regulatory T cells in brain tissue and thus exerts a neuroprotective effect.\textsuperscript{[22]} OX40L-OX40 can inhibit the differentiation and activity of regulatory T cells.\textsuperscript{[23]} Second, ischemic stroke triggers sustained and stable changes in the systemic immune system, including specific lymphopenia, excessive production of IL-10, and prolonged Th2 biased immunity, all of which lead to severe immunosuppression in stroke patients.\textsuperscript{[24]} The OX40L-OX40 pathway could enhance Th2 responses, and this indicates that patients with high serum OX40L have high likelihood to develop low immune function and are prone to various infections.\textsuperscript{[23]} In this article, non-survivors of CI patients showed significantly higher rate of pulmonary infection than survivors. Our results are also supported by a previous report that subjects with higher OX40L expression on bronchiolar progenitors had increased susceptibility to influenza infection and pneumonia.\textsuperscript{[26]} However, the contribution of OX40L to pulmonary infection is still unclear and deserves further investigation.

The limitations of this study are as follows. First, the source of serum OX40L in patients with CI is still unclear. Further study is required to explore whether it is derived from peripheral blood lymphocytes. Second, in this study, only serum OX40L levels in patients with CI at the beginning of admission were measured, and detailed time series of serum OX40L levels after admission was lacking. Third, an animal model is needed to elucidate the possible pathological mechanisms of OX40L on ischemic brain tissue.

In conclusion, serum OX40L levels are elevated in patients with CI, and elevated in nonsurvivors than in survivors. There was a correlation between serum OX40L levels and mortality in patients with CI.

Author contributions

Lun-Lin Mao performed the experiments and wrote the manuscript; Wen-Ya Chen designed the study and revised the manuscript; Ai-Jin Ma performed the experiments; Li-Li Ji collected and analyzed clinical data; Ting-Ting Huang collected laboratory variables of patients and performed statistical analysis.

Conceptualization: Wen-Ya Chen.

Data curation: Li-Li Ji, Ting-Ting Huang.

Investigation: Lun-Lin Mao, Ai-Jin Ma.

Project administration: Wen-Ya Chen.

Software: Li-Li Ji, Ting-Ting Huang.

Writing – original draft: Lun-Lin Mao.

Writing – review & editing: Wen-Ya Chen.

References

\textsuperscript{[1]} Liu L, Wang D, Wong KS, et al. Stroke and stroke care in China: huge burden, significant workload, and a national priority. Stroke 2011; 42:3651–4.

\textsuperscript{[2]} Khoshnam SE, Winlow W, Farzaneh M, et al. Pathogenic mechanisms following ischemic stroke. Neurol Sci 2017;38:1167–86.

\textsuperscript{[3]} Świtońska M, Slomka A, Korbal P, et al. Association of neutrophil-to-lymphocyte ratio and lymphocyte-to-monocyte ratio with treatment modalities of acute ischaemic stroke: a pilot study. Medicina (Kaunas) 2019;55:pii: E342.

\textsuperscript{[4]} Slomka A, Świtońska M, Sinkiewicz W, et al. Haemostatic factors do not account for worse outcomes from ischaemic stroke in patients with higher C-reactive protein concentrations. Ann Clin Biochem 2017;54:378–85.

\textsuperscript{[5]} Dawson DA, Martin D, Hallenbeck JM. Inhibition of tumor necrosis factor-alpha reduces focal cerebral ischemic injury in the spontaneously hypertensive rat. Neurosci Lett 1996;218:41–4.

\textsuperscript{[6]} McCombe PA, Read SJ. Immune and inflammatory responses to stroke: good or bad? Int J Stroke 2008;3:254–63.

\textsuperscript{[7]} Vila N, Castillo J, Dávalos A, et al. Levels of anti-inflammatory cytokines and neurological worsening in acute ischemic stroke. Stroke 2003;34:671–5.

\textsuperscript{[8]} Webb GJ, Hirschfield GM, Lane PJ. OX40, OX40L and autoimmunity: a comprehensive review. Clin Rev Allergy Immunol 2016;50:312–32.

\textsuperscript{[9]} Wang YH, Liu YJ. Thymic stromal lymphopoeitin, OX40-ligand, and interleukin-25 in allergic responses. Clin Exp Allergy 2009;39:798–806.

\textsuperscript{[10]} Zhang JY, Liu B, Wang YN, et al. Effect of rosuvastatin on OX40L and PPAR-expression in human umbilical vein endothelial cells and atherosclerotic cerebral infarction patients. J Mol Neurosci 2014;52:261–8.

\textsuperscript{[11]} Tesdale G, Bennett J. Assessment of coma and impaired consciousness. A practical scale. Lancet 1974;2:81–4.

\textsuperscript{[12]} Liu B, Yu G, Yang Z, et al. Simvastatin reduces OX40 and OX40 ligand expression in human peripheral blood mononuclear cells and in patients with atherosclerotic cerebral infarction. J Int Med Res 2009;37:601–10.

\textsuperscript{[13]} Inagaki-Katahisa N, Ito T, Inaba M, et al. Statins can suppress DC-mediated Th2 responses through the repression of OX40-ligand and CCL17 expression. Eur J Immunol 2019;49:2031–62.

\textsuperscript{[14]} Sun P, Hernandez-Guillamon M, Campos-Martorell M, et al. Simvastatin blocks soluble SSAO/VAP-1 release in experimental models of cerebral ischemia: possible benefits for stroke-induced inflammation control. Biochim Biophys Acta 2018;1864:542–33.

\textsuperscript{[15]} Ezzat MH, Imam SS, Shaheen KY, et al. Serum OX40 ligand levels in asthmatic children: a potential biomarker of severity and persistence. Allergy Asthma Proc 2011;32:313–8.

\textsuperscript{[16]} Yan J, Gong J, Chen G, et al. Evaluation of serum soluble OX40 ligand as a prognostic indicator in acute coronary syndrome patients. Clin Chim Acta 2010;411:1662–5.

\textsuperscript{[17]} Lin Y, Zhang L, Dai Y, et al. Expression of interleukin-9 and its upstream stimulating factors in rats with ischemic stroke. Neurol Sci 2015;36:913–20.

\textsuperscript{[18]} Snarska K, Kapica-Topczewska K, Bachorzewska-Gajewska H, et al. Renal function predicts outcomes in patients with ischaemic stroke and haemorrhagic stroke. Kidney Blood Press Res 2016;41:424–33.

\textsuperscript{[19]} Farres MN, Al-Ziffaf DS, Aly AA, et al. OX40/OX40L in systemic lupus erythematosus: association with disease activity and lupus nephritis. Ann Saudi Med 2011;31:29–34.

\textsuperscript{[20]} Shu JZ, Wang L, Zhu Y, et al. OX40 ligand levels and high-sensitivity C-reactive protein levels in blood from local coronary plaque and the femoral artery in patients with acute coronary syndrome or stable angina. J Int Med Res 2011;39:1275–83.

\textsuperscript{[21]} Rodríguez-Pere Al, Gutierrez-Vargas J, Cardona-Gómez GP, et al. Atorvastatin modulates regulatory T cells and attenuates cerebral damage in a model of transient middle cerebral artery occlusion in rats. J Neuroimmune Pharmacol 2017;12:152–62.

\textsuperscript{[22]} Brea D, Aguila J, Rodríguez-Yáñez M, et al. Regulatory T cells modulate inflammation and reduce infarct volume in experimental brain ischaemia. J Cell Mol Med 2014;18:1571–9.

\textsuperscript{[23]} Croft M, So T, Duan W, et al. The significance of OX40 and OX40L to T-cell biology and immune disease. Immunol Rev 2009;229:173–91.

\textsuperscript{[24]} Wong CH, Jenne CN, Tam PP, et al. Prolonged activation of invariant natural killer T cells and TH2-skewed immunity in stroke patients. Front Neurosci 2017;8:66.

\textsuperscript{[25]} Wu Q, Tang Y, Hu X, et al. Regulation of Th1/Th2 balance through OX40/OX40L signalling by glycyrrhizic acid in a murine model of asthma. Respirology 2016;21:102–11.

\textsuperscript{[26]} Hirano T, Kikuchi T, Tode N, et al. OX40 ligand newly expressed on bronchiolar progenitors mediates influenza infection and further exacerbates pneumonia. EMBO Mol Med 2016;8:422–36.