Original Article

Link between Lipoprotein-Associated Phospholipase A_2 Gene Expression of Peripheral-Blood Mononuclear Cells and Prognostic Outcome after Acute Ischemic Stroke

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***Aim*: To evaluate the potential of the lipoprotein-associated phospholipase A_2 (Lp-PLA_2) level as a biomarker in the prediction of prognostic outcome in patients with acute ischemic stroke (IS).**
***Methods*: From October 2008 to March 2010, 130 patients with acute IS were prospectively enrolled in the study and their medical records were reviewed. A blood sample was collected from each patient 48 hours after acute IS, as well as from 20 healthy volunteers as controls. Messenger-RNA (mRNA) expression of Lp-PLA_2 of peripheral-blood mononuclear cells (PBMNCs) relative to that of β actin was measured using quantitative reverse transcription polymerase chain reaction (RT-PCR).**
***Results*: Patients with acute IS exhibited significantly higher Lp-PLA_2 mRNA expression of PBMNCs than the control group ($p<0.0001$). Lp-PLA_2 mRNA expression of PBMNCs in patients with a major adverse clinical outcome (MACO) (defined as recurrent stroke or death) within 90 days was significantly higher than in patients without MACO ($p=0.006$). Furthermore, elevated Lp-PLA_2 mRNA expression was strongly associated with old age, diabetes mellitus, a positive history of significant coronary arterial disease and significant stenosis of the extra-cranial carotid arteries (all $p<0.04$), and positively correlated with the body mass index, leukocyte count, and serum levels of total cholesterol and low-density lipoprotein cholesterol. Multivariate analysis revealed that Lp-PLA_2 mRNA expression of PBMNCs was a significant independent predictor of MACO within 90 days ($p=0.011$).**
***Conclusion*: Elevated Lp-PLA_2 mRNA expression of PBMNCs seems to be a potential biomarker for predicting an unfavorable outcome in patients with acute IS.**

J Atheroscler Thromb, 2012; 19:523-531.

Key words; Acute ischemic stroke, Lipoprotein-associated phospholipase A_2 activity, Clinical outcome

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Received: September 19, 2011
Accepted for publication: November 24, 2011

Introduction

Many studies have reported that inflammation might be involved in endothelial dysfunction and propagation of atherosclerosis, which may eventually lead to acute coronary syndrome and ischemic stroke[1-5]. Besides the assessment of traditional risk

factors, many inflammatory biomarkers in blood or damaged atherosclerotic plaque are useful for assessing the potential risk of cardiovascular diseases, monitoring disease propagation, and predicting the outcome in various clinical settings[1-10]. Of these biomarkers[1-10], lipoprotein-associated phospholipase A_2 (Lp-PLA_2) has recently been described as a novel biomarker which is strongly associated with atherosclerosis-related inflammatory processes and plaque instability in histopathologic studies of the coronary and carotid arteries[10-12].

Lp-PLA_2, also known as platelet-activating factor acetylhydrolase, is produced predominantly by monocyte-derived macrophages, T-lymphocytes and mast cells[13-15]. Many studies have demonstrated that Lp-PLA_2 is an enzyme with broad capabilities, allowing cleavage of oxidized fatty acids at the sn-2 position of oxidized phospholipids and the generation of lysophosphatidylcholine, free oxidized fatty acids and bioactive proatherogenic lipids[10, 16]. Several basic and clinical researches have advocated that Lp-PLA_2 may be a biological effector and biomarker associated with the production of low-density lipoprotein cholesterol (LDL-C), metabolic syndrome, atherosclerotic and cardiovascular diseases[17-21]. Although Lp-PLA_2 activity has been reported as an independent predictor of the long-term prognostic outcome in patients with or without cardiovascular diseases[10, 17, 21-23], whether this biomarker can also be applied in patients with acute ischemic stroke (IS) has not been well addressed[8, 24]. The purpose of this study was to verify the potential of Lp-PLA_2 mRNA expression in peripheral-blood mononuclear cells (PBMNCs) as a biomarker in the prediction of prognostic outcome in patients with acute IS.

Materials and Methods

Patient Enrollment and Exclusion Criteria

This study was approved by the Institutional Review Committee on Human Research of the Chang Gung Memorial Hospital (approval number: 96-1381A) and was conducted at Kaohsiung Chang Gung Memorial Hospital.

Acute IS was defined as the sudden onset of loss of global or focal cerebral function persisting for more than 24 hours. The neurologic imaging criteria for the diagnosis of acute IS included new focal or diffuse low attenuation areas in the brain on computed tomography or the presence of a high signal intensity area on diffusion-weighted imaging (DWI) and a low signal intensity area on the apparent diffusion coefficient (ADC) map on magnetic resonance (MR) studies. The degree of neurological impairment was assessed by neurologists based on the National Institutes of Health Stroke Scale (NIHSS).

Patients of all ages with acute IS were eligible for enrollment in the current study. Inclusion criteria included a total NIHSS score >2 and a time window of ≤ 48h from the onset of symptoms to the time point of the collection of blood samples (48h after IS). Exclusion criteria included patients with contraindications for MR imaging, lack of evidence of acute IS on MR studies, the presence of intracranial hemorrhage, major surgery or trauma within the preceding 3 months, concurrent liver function abnormality, hematological disorders, malignancy, febrile disorders, acute or chronic inflammatory diseases, atrial fibrillation, pregnancy, or thrombolytic therapy.

From October 2008 to March 2010, 130 patients presenting with acute IS who fulfilled the inclusion criteria were enrolled for blood sampling to measure Lp-PLA_2 mRNA expression of PBMNCs. Twenty age- and gender-matched healthy volunteers were also recruited as the control group. Informed consent was obtained from all the patients and volunteers.

Imaging Studies and Laboratory Investigations

In addition to clinical assessments, other studies, including a chest radiograph, brain computed tomography and/or MR imaging, duplex scanning of the carotid arteries, 12-lead electrocardiography, and echocardiography, were performed. White blood cell (WBC) count and biochemical data were acquired on admission.

Protocol for RNA Extraction

Lysis/binding buffer (400 μL) (High Pure RNA Tissue Kit; Roche, Germany) and an appropriate amount of frozen PBMNCs were added to a nuclease-free 1.5 mL microcentrifuge tube, followed by disruption and homogenization of BPMNCs using a rotor-stator homogenizer (Roche). The lysate in the microcentrifuge tube was then centrifuged for two minutes at 13,000g. Only the supernatant was utilized for subsequent steps. Absolute ethanol (200 μL) was then added to the lysate supernatant and mixed well. The entire sample in the upper reservoir was pipetted into a High Pure filter tube (Roche) that was placed in a collection tube (Roche). This sample was then centrifuged for 30 seconds at 13,000g in a standard tabletop microcentrifuge. The filter tube was removed from the collection tube and the flowthrough liquid was discarded. For each isolation, 90 μL DNase incubation buffer was pipetted into a sterile 1.5 μL reaction tube, 10 μ of DNase I working solution was then added,

Table 1. Baseline Characteristics of Ischemic Stroke and Normal Control Groups

Variables	Study patients (n=130)	Normal control (n=20)	p* value
Age (yrs)	65.4±12.4	64.6±8.2	0.692
Male gender	67.7% (88)	60% (12)	0.671
WBC count (×10^3/uL)	8.5±2.9	6.5±1.6	0.001
Total cholesterol level (mg/dL)	183±46	195±34	0.264
HDL-C (mg/dL)	47±15	58±16	0.004
LDL-C (mg/dL)	111±35	112±31	0.867
Creatinine (mg/dL)	1.04±0.51	0.86±0.23	0.011
Lp-PLA2 mRNA†	3.1 (2.0-5.2)	1 (1-1)	<0.001
SBP (mmHg)	142±24	140±16	0.657
DBP (mmHg)	82±14	77±11	0.674
Significant ECCA stenosis (%)‡	14.6% (19)	–	
Statin therapy	33.1% (43)	–	
ACEI/ARB therapy	41.5% (54)	–	
Recurrent stroke	7.7% (10)	–	
Mortality within 90 days	3.1% (4)	–	
Combined MACO	10.8% (14)		

Data are expressed as the mean±SD or % (No.) of patients.
ACEI/ARB=angiotensin converting enzyme inhibitor/angiotensin Ⅱ type Ⅰ receptor blocker; DBP=diastolic blood pressure; ECCA=extra-cranial carotid artery; LDL-C=low-density lipoprotein cholesterol; HDL-C=high-density lipoprotein cholesterol; Lp-PLA2=lipoprotein-associated phospholipase A2; MACO=major adverse clinical outcome (defined as recurrent stroke or death within 90 days); SBP=systolic blood pressure; WBC=white blood cell.
*by Chi-square test or Fisher's exact test for categorical data; by t-test or Mann-Whitney U test for continuous data.
† Indicated mRNA expression of Lp-PLA2 of peripheral-blood mononuclear cells which was presented as the median (Interquartile range) level.
‡ defined as ECCA stenosis ≥ 50% regardless of anatomical locations by carotid Doppler examination.

mixed and incubated for 15 minutes at 25℃. Wash buffer Ⅰ (500 μL) was then added to the upper reservoir of the filter tube, which was then centrifuged for 15 seconds at 8,000g. The filter tube was removed from the collection tube and the flowthrough liquid was then discarded. Wash buffer Ⅱ (500 μL) was added to the upper reservoir of the filter tube, which was then centrifuged for 15 seconds at 8,000g and the flowthrough was discarded. Wash buffer Ⅱ (300 μL) was added to the upper reservoir of the filter tube, which was centrifuged for 2 minutes at full-speed, approximately 13,000g. The column was then carefully removed from the collection tube such that the column did not contact the flowthrough, to avoid ethanol carryover. The filter tube was then inserted into a 1.5 mL nuclease-free and sterilized microcentrifuge tube. Elution buffer (100 μL) was added to the upper reservoir of the filter tube and the tube assembly was then centrifuged for 1 minute at 8,000g, resulting in eluted RNA in the microcentrifuge tube.

Reverse Transcription qPCR Analysis for Relative mRNA Expression of Lp-PLA2 of PBMNCs to β Actin

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was conducted using Light-Cycler TaqMan Master (Roche) in a single capillary tube according to the manufacturer's guidelines for individual component concentrations. The Lp-PLA2 forward (TGGCTTACCTTAGAACCCTGA) and reverse (TTTTGCTCTTTGCCGTACCT) primers were each designed based on individual exons of the target gene sequence to avoid amplifying genomic DNA.

During PCR, the probe was hybridized to its complementary single-strand DNA sequence within the PCR target. As amplification occurred, the probe was degraded due to the exonuclease activity of Taq DNA polymerase, thereby separating the quencher from the reporter dye during extension. During the entire amplification cycle, light emission increased exponentially. A positive result was determined by identifying the threshold cycle value at which reporter dye emission appeared above the background.

Table 2. Comparison of Baseline Characteristics between Patients with and without 90 Days MACO

Variables	With MACO (n=14)	Without MACO (n=116)	p^* value
Age (yrs)	65.9 ± 11.4	65.4 ± 13.2	0.893
Male gender	85.7% (12)	65.5% (76)	0.221
Hypertension	78.6% (11)	69.8% (81)	0.713
Diabetes mellitus	42.9% (6)	31.9% (37)	0.601
Current smoking	50% (7)	25.9% (30)	0.115
Previous stroke by history	57.1% (8)	30.2% (35)	0.084
Previous stroke by MRI	71.4% (10)	61.7% (71)	0.678
NIHSS at admission	8.21 ± 6.46	7.86 ± 8.97	0.461
WBC count ($\times 10^3$/mL)	8.9 ± 2.5	8.5 ± 2.9	0.591
Total cholesterol level	191 ± 45	182 ± 46	0.456
HDL-C (mg/dL)	40 ± 10	47 ± 16	0.104
LDL-C (mg/dL)	112 ± 41	111 ± 35	0.998
Creatinine (mg/dL)	1.37 ± 0.50	1.01 ± 0.50	0.010
Lp-PLA$_2$ mRNA †	6.73 (3.28-10.67)	2.99 (1.98-4.48)	0.006
BMI (kg/m^2)	25.6 ± 4.4	24.3 ± 4.1	0.542
HbA1c (%)	6.6 ± 1.2	6.8 ± 2.2	0.447
SBP (mmHg)	145 ± 16	142 ± 25	0.527
DBP (mmHg)	81 ± 10	82 ± 14	0.830
Significant ECCA stenosis ‡	7.1% (1)	15.5% (18)	0.662
Statin therapy	35.7% (5)	33.0% (38)	0.841
ACEI/ARB therapy	50% (7)	40.7% (47)	0.694

Data are expressed as the mean ± SD or % (No.) of patients.
ACEI/ARB = angiotensin converting enzyme inhibitor/angiotensin Ⅱ type Ⅰ receptor blocker; Lp-PLA$_2$ = lipoprotein-associated phospholipase A$_2$; BMI = body mass index; DBP = diastolic blood pressure; NIHSS = National Institutes of Health stroke scale; ECCA = extra-cranial carotid artery; HbA1c = hemoglobin A1c; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; WBC = white blood cell.; MACO = major adverse clinical outcome (defined as recurrent stroke or death within 90 days).
* by Chi-square test or Fisher's exact test for categorical data; by t-test or Mann-Whitney U test for continuous data.
† Indicated mRNA expression of Lp-PLA$_2$ of peripheral-blood mononuclear cells which was presented as the median (Interquartile range) level.
‡ defined as ECCA stenosis ≥ 50% regardless of anatomical locations by carotid Doppler examination.

Medications

Aspirin was the first choice for our patients with acute IS except for patients who could not tolerate such treatment due to aspirin-related peptic ulcer or upper gastrointestinal tract bleeding. For these patients, Clopidogrel was administered. Other commonly used drugs included statins, angiotensin converting enzyme inhibitors, calcium channel blocking agents, and beta blockers.

Definitions of Major Adverse Clinical Outcome and Extra-Cranial Carotid Artery Stenosis

The major adverse clinical outcome (MACO) was defined as the occurrence of recurrent IS or death within 90 days during follow-up. Significant stenosis of the extra-cranial carotid artery (ECCA) was defined as stenosis ≥ 50%, regardless of anatomical location, as revealed on carotid Doppler examination.

Statistical Analysis

Continuous variables with normal distribution were expressed as the mean ± SD and those without normal distribution were presented as median values (interquartile interval). Categorical data were analyzed by the Chi-square test or Fisher's exact test for categorical data and continuous variables were analyzed using the unpaired t-test or the Mann-Whitney U test where appropriate. The predictive values of categorically variables for elevated Lp-PLA$_2$ mRNA expression of PBMNCs were assessed with the logistic regression test. Spearman's rank test was used to assess the correlations between quantitative variables without normal distribution. Multiple stepwise regression analysis was utilized to assess the independent predictors of

MACO within 90 days. Statistical analysis was performed using SPSS statistical software for Windows version 13 (SPSS for Windows, version 13; SPSS Inc., IL, USA). $P<0.05$ was considered significant.

Results

Baseline Characteristics of Acute Ischemic Stroke Patients and Healthy Controls (Table 1)

Patients with acute IS and healthy controls showed no significant differences with respect to age, gender, systolic and diastolic blood pressure, serum levels of total cholesterol and LDL-C; however, the WBC count, serum level of creatinine and Lp-PLA2 mRNA expression were significantly higher, whilst the serum level of high-density lipoprotein cholesterol (HDL-C) was significantly lower in patients with acute IS than in healthy controls. The percentages of patients with recurrent stroke and death within 90 days were 7.7% and 3.1%, respectively, and the percentage of MACO was 10.8%.

Comparison of Baseline Characteristics between Patients with and without MACO (Table 2)

Baseline characteristics and Lp-PLA2 mRNA expression of PBMNCs in patients with MACO ($n=14$) and without MACO ($n=116$) were compared. There were no significant differences in terms of age, gender, systolic and diastolic blood pressure, the presence of coronary artery disease (CAD) risk factors, positive previous stroke history, positive findings of previous stroke on MR imaging, and NIHSS score on admission between patients with and without MACO. Furthermore, there were no significant differences between the two groups with respect to the serum levels of total cholesterol, LDL-C, HDL-C, hemoglobin A1c, WBC count, body-mass-index (BMI), the frequencies of associated significant stenosis of ECCA and prior usage of statins, angiotensin converting enzyme inhibitors and angiotensin II type I inhibitors. In contrast, the serum level of creatinine and Lp-PLA2 mRNA expression of PBMNCs were significantly higher in patients with than without MACO.

Predictors of Elevated Lp-PLA2 mRNA Expression of PBMNCs (Table 3)

The logistic regression test revealed that older age (≥65 years old), diabetes mellitus (DM), a positive past history of significant coronary artery obstruction treated by percutaneous coronary intervention (PCI) and the presence of significant ECCA stenosis were strongly associated with elevated Lp-PLA2 mRNA expression of PBMNCs.

Table 3. Predictors of Increased Lp-PLA2 mRNA Expression in Peripheral Blood Mononuclear Cells

Variables	Number	Lp-PLA2	p^* value
Age			
≥65 yrs	67	3.17 (2.01-6.21)	0.031
<65 yrs	63	2.67 (1.81-4.01)	
Gender			
male	88	3.02 (2.02-4.26)	0.575
female	42	3.27 (1.95-6.26)	
Risk factor			
DM (positive)	43	3.93 (2.88-6.75)	0.008
DM (negative)	87	2.78 (1.91-4.21)	
HTN (positive)	92	3.14 (2.12-5.52)	0.216
HTN (negative)	38	2.83 (1.86-5.21)	
CAD (positive)†	11	6.15 (3.61-9.44)	0.009
CAD (negative)	119	2.99 (1.98-4.94)	
Af (positive)	12	2.31 (1.79-6.66)	0.747
Af (negative)	118	3.01 (1.98-4.64)	
ECCA stenosis			
≥50%	19	5.19 (2.63-10.31)	0.014
<50%	111	3.01 (1.98-4.27)	
Stroke severity			
NIHSS ≥8	57	3.09 (2.07-5.47)	0.593
NIHSS <8	73	3.02 (1.96-4.41)	

Lp-PLA2 = lipoprotein-associated phospholipase A2; DM = diabetes mellitus; HTN = hypertension; CAD = coronary artery disease (defined as epicardial coronary artery stenosis ≥50% by coronary angiographic examination); Af = atrial fibrillation; ECCA = extra-cranial carotid artery; NIHSS = National Institutes of Health Stroke Scale.

*by Mann-Whitney *U* test for continuous data presented as the median (interquartile range).

†indicated patients with a past history of significant coronary artery disease treated by percutaneous coronary intervention (PCI).

Correlation between Continuous Variables and Elevated Lp-PLA2 mRNA Expression of PBMNCs (Fig. 1)

Spearman's rank correlation test revealed that BMI, WBC count, the serum levels of total cholesterol and LDL-C had significant positive correlations with elevated Lp-PLA2 mRNA expression of PBMNCs (all $p<0.05$).

Univariate and Multivariate Analyses for Predictors of MACO within 90 Days (Tables 4 and 5)

Univariate analysis showed that only an elevated serum level of creatinine and Lp-PLA2 mRNA expression of BPMNCs were significantly predictive of MACO within 90 days and multiple stepwise logistic regression analysis further confirmed that these two parameters were independent and significant predictors of the occurrence of MACO within 90 days.

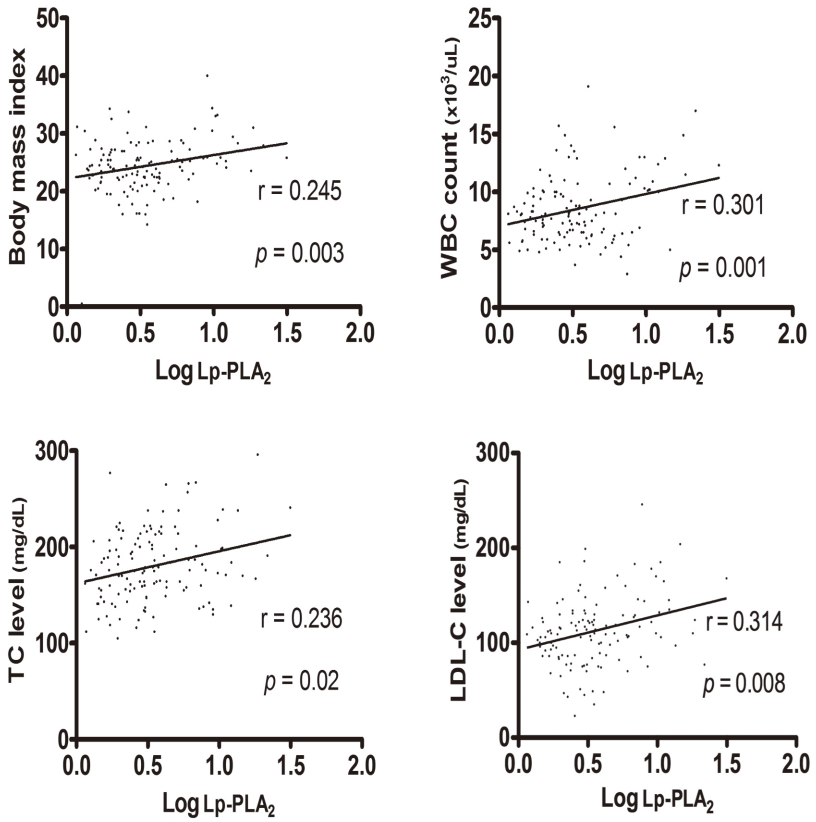

Fig. 1. Spearman's rank test for assessing the correlation between Log lipoprotein-associated phospholipase A_2 (Lp-PLA_2) mRNA expression and body mass index ($p=0.003$, $r=0.245$), white blood cell (WBC) count ($p=0.001$, $r=0.301$) and the levels of total cholesterol (TC) ($p=0.02$, $r=0.236$) and low-density lipoprotein cholesterol (LDL-C) level ($p=0.008$, $r=0.314$).

Discussion

The link between elevated Lp-PLA_2 activity and the risks of cardiovascular or cerebrovascular events has been investigated in several studies[25-27]. The present study with at least 90 days follow-up of 130 consecutive patients presented with acute IS showed that Lp-PLA_2 mRNA expression of PBMNCs was significantly higher in patients with acute IS than in healthy controls.

Of note, we found that old age and the presence of DM were strongly predictive of elevated Lp-PLA_2 mRNA expression of PBMNCs. Both old age and DM are well known traditional risk factors for the development of endothelial dysfunction and atherosclerosis[28, 29]. Therefore, we postulate that elevated Lp-PLA_2 activity may also play a role in the formation and propagation of atherosclerotic plaques that may lead to occlusive arterial changes.

Lp-PLA_2 has been reported as a proatherogenic factor which may promote the generation of oxidized LDL-C and free fatty acid with subsequent direct participation in the formation and rupture of carotid plaques[10, 12, 15, 18, 19]. Our results also revealed a strong association between significant coronary artery or ECCA stenosis and elevated Lp-PLA_2 mRNA expression of PBMNCs. Moreover, significant positive correlations between enhanced Lp-PLA_2 mRNA expression and serum levels of total cholesterol and LDL-C, WBC count and BMI were also identified. The levels of total cholesterol and LDL-C have been documented as two important risk factors of atherosclerotic cardiovascular diseases[30, 31]. On the hand, a high WBC count and BMI are also suggestive of the presence of inflammatory changes and higher chances of an association with metabolic syndrome, respectively. Concurring with previous studies[28-31], our results further verify that elevated Lp-PLA2 activity may be a useful biomarker of proatherogenic and inflammatory changes[10] that may associate with endothelial dys-

Table 4. Univariate Logistic Regression Analysis of Predictors for Combined 90 Days MACO after Ischemic Stroke

Variables	Odds Ratio	95% CI	*p* value
Creatinine level	2.852	1.172-6.938	0.021
High-density lipoprotein	0.958	0.910-1.008	0.096
Log Lp-PLA2 mRNA expression	19.463	3.193-120.839	0.001
Old stroke by history	3.086	0.996-9.555	0.051

MACO = major adverse clinical outcome (defined as recurrent stroke or death within 90 days); CI = confidence interval; Lp-PLA2 = lipoprotein-associated phospholipase A2

Table 5. Multiple Stepwise Logistic Regression Analysis of Predictors for Combined 90-Day MACO after Ischemic Stroke

Variables	Odds Ratio	95% CI	*p* value
Creatinine (mg/dL)	3.521	1.234-10.052	0.019
Log Lp-PLA2 mRNA expression	34.851	4.219-287.884	0.001

MACO = major adverse clinical outcome (defined as recurrent stroke or death within 90 days); CI = confidence interval; Lp-PLA2 = lipoprotein-associated phospholipase A2

function and the development of atherosclerosis.

Although Elkind *et al*. have reported that stroke patients with high Lp-PLA2 activity were predisposed to recurrence after the first IS[8,24], comprehensive data in this clinical setting remain limited. In the present study, multiple stepwise logistic regression analysis confirmed that elevated Lp-PLA2 mRNA expression of PBMNCs is a significant independent predictor of MACO within 90 days. Furthermore, our results also disclosed that an elevated serum level of creatinine is another significant independent predictor of MACO in acute IS patients. Previous studies have described that renal insufficiency is an important risk factor for predicting an untoward clinical outcome in patients with acute coronary syndrome undergoing PCI[32-34]. However, the relationship between the serum level of creatinine and clinical outcome of stroke patients has not been addressed. To our knowledge, this is the first report of applying an elevated serum level of creatinine as a novel independent predictor of MACO within 90 days in patients with acute IS.

In conclusion, Lp-PLA2 mRNA expression of PBMNCs was markedly elevated in patients with acute IS. It is noteworthy that enhanced mRNA expression of this enzyme in PBMNCs could be considered a useful biomarker for predicting an unfavorable outcome and as an independent predictor of MACO within 90 days in patients with acute IS. Therefore, this novel inflammatory biomarker may be helpful for the stratification of acute IS patients into high-risk and lower-risk subgroups in clinical practice.

Study Limitations

The present study utilized RT-PCR rather than ELISA to measure the level of Lp-PLA2 and could only reflect the mRNA expression level of this enzyme in leukocytes; therefore, the details of the activity or the level of related protein in the circulation were not investigated.

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