Plasma Concentration of SCUBE1, a Novel Platelet Protein, Is Elevated in Patients With Acute Coronary Syndrome and Ischemic Stroke

Dao-Fu Dai, MD,*† Peterus Thajeb, MD,‡§ Cheng-Fen Tu, MS,¶ Fu-Tien Chiang, MD, PhD,* Chien-Hsiun Chen, PhD,¶ Ruey-Bing Yang, PhD,¶ Jin-Jer Chen, MD*¶
Taipei, Taiwan; and Manoa, Hawaii

Objectives
This study investigates the potential application of plasma SCUBE1 [signal peptide–CUB (complement C1r/C1s, Uegf, and Bmp1)–EGF (epidermal growth factor)-like domain-containing protein 1] as a biomarker of platelet activation in acute coronary syndrome (ACS) and acute ischemic stroke (AIS).

Background
Platelet activation plays a crucial role in ACS and AIS. Platelet stimulation is associated with increased plasma concentration of SCUBE1, a novel platelet-endothelial secreted protein identified in our previous study.

Methods
Plasma concentrations of SCUBE1 from 40 ACS and 40 AIS patients were measured by enzyme-linked immunosorbent assay and compared with the levels of 40 healthy control subjects and 83 chronic coronary artery disease (CAD) patients. Two-dimensional electrophoresis followed by Western blotting was used to characterize SCUBE1 protein in patients’ plasma.

Results
Plasma SCUBE1 concentration was virtually undetectable in healthy control subjects and CAD patients, but was significantly higher in ACS and AIS patients (median 205 and 95.1 ng/ml, respectively, p < 0.01). The increase in plasma SCUBE1 was detectable in the plasma as early as 6 h after the onset of symptoms and remained detectable up to 84 h. Plasma SCUBE1 concentration is an independent predictor of stroke severity based on National Institutes of Health Stroke Scale (β = 3.18, p < 0.001). Furthermore, smaller SCUBE1 fragments were detected in ACS patients’ plasma, suggesting that plasma SCUBE1 might subject to a proteolytic regulation under pathological conditions.

Conclusions
Plasma SCUBE1 concentration is significantly elevated in ACS and AIS but not CAD patients. Plasma SCUBE1 is a potential biomarker of platelet activation in acute thrombotic disease. (J Am Coll Cardiol 2008;51:2173–80) © 2008 by the American College of Cardiology Foundation

We have previously identified a novel family of secreted, surface-anchored proteins by various genomic approaches (1,2). These proteins harbor an array of signal peptide, complement proteins C1r/C1s, Uegf, and Bmp1 (CUB), and epidermal growth factor (EGF)-like domains and hence are termed SCUBEs. Three distinct members of SCUBE, namely SCUBE1 to 3, have been described in human, mouse, and zebrafish (3–6). They share common protein domains, consisting of an amino-terminal signal peptide, 9 copies of EGF-like repeats, a spacer region with multiple potential N-glycosylation sites, and a CUB domain at the carboxyl terminus (1,2).

We and others have shown that SCUBE1 is expressed during mouse development as well as in adult endothelial cells (1,2,7). When overexpressed in human embryonic kidney (HEK)-293T cells, recombinant SCUBE1 protein is a secreted glycoprotein that forms oligomers tethered to the cell surface (1). Our recent study found that SCUBE1 is highly expressed in platelets, at a level higher than in endothelial cells. These molecules are stored within the

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α-granules of inactive platelets, translocated to the platelet surface upon activation by thrombin, proteolytically released as smaller soluble fragments, and incorporated into thrombus (8). Furthermore, immunohistochemistry revealed the deposition of SCUBE1 in subendothelial matrix of human advanced atherosclerotic lesions. Since EGF-like repeats are well known in mediating adhesive interactions (9,10), our studies demonstrated that the amino-terminal EGF-like repeats of SCUBE1 are able to enhance platelet adhesion as well as ristocetin-induced platelet agglutination. Thus, SCUBE1 might function as a novel platelet-endothelial adhesion molecule and play pathological roles in cardiovascular biology (8).

Platelet activation and aggregation are well recognized as primary reactions in arterial thrombosis and, accordingly, are responsible for the ischemic complications of acute coronary syndromes (ACS) (11,12) and acute ischemic stroke (AIS) (13,14). Acute coronary syndromes are initiated by plaque rupture or erosion, with subsequent platelet activation and thrombus formation. The importance of platelet activation in the pathogenesis of these atherothrombotic complications was definitely shown by the application of antiplatelet therapy as the most important management in ACS and AIS patients (12,15–18).

We hypothesize that plasma SCUBE1 could be a good biomarker of platelet activation in acute atherothrombotic diseases. The present study was designed to compare the plasma concentrations of SCUBE1 in normal control subjects, in patients with chronic coronary artery disease (CAD), ACS, and AIS. In addition, biochemical characterization of plasma SCUBE1 protein was performed.

**Methods**

This study was approved by institutional review boards of our hospitals, and written informed consents were obtained from the participants. The investigation conforms with principles outlined in the Declaration of Helsinki (19).

**Study population.** We recruited 83 subjects with chronic CAD and 40 subjects with ACS from 152 consecutive patients undergoing cardiac catheterization, as well as 40 of 46 consecutive patients with AIS from 2 medical centers in Taiwan. For control subjects, we recruited 40 healthy subjects visiting for health check-up, frequency matched for gender and age to the ACS patients. During hospitalization, all participants had a record of medical history of diabetes, hypertension, smoking, hypercholesterolemia (total cholesterol ≥200 mg/dl and/or low-density lipoprotein cholesterol ≥160 mg/dl), prior antiplatelet and statin use.

**Recruitment and exclusion criteria.** Four groups of subjects were studied. Group I (CAD) included 83 subjects with chronic stable angina and angiography-proven CAD, defined as at least 1 coronary artery stenosis of >50% luminal diameter narrowing. Group II (ACS) included 40 patients with ST-segment elevation myocardial infarction (STEMI), non–ST-segment elevation myocardial infarction (NSTEMI), or high-risk unstable angina (UA) within 120 h of onset. ST-segment elevation myocardial infarction and NSTEMI were defined according to the consensus of the American Heart Association/American College of Cardiology. Unstable angina was defined as Braunwald class III chest pain with significant electrocardiogram changes, including transient ST-segment depression or T inversion.

Group III (AIS) consisted of 40 patients with first acute cerebral infarction within 120 h of onset, including large-vessel atherothrombotic (LAT) stroke and small lacunar infarction. Acute ischemic stroke was defined as acute presentation of focal neurological deficits in concordance with ischemic changes at corresponding areas by diffusion weighted image and T2-weighted image of the brain magnetic resonance imaging. Large-vessel atherothrombotic ischemic stroke was defined as acute cerebral infarction involving the trunk or branched vessels of the major cerebral arteries, whereas acute lacunar infarction was defined as small infarction (<20 mm²) in the areas supplied by the penetrating arterioles (20–22). Six AIS patients were excluded because of evidence of old infarctions in 3 patients, hemorrhagic transformation of the infarctions in 2, and co-existence of chronic subdural hemorrhage in the others. To assess the severity of AIS, we evaluated the baseline National Institutes of Health Stroke Scale (NIHSS) (23). Group IV (control subjects) included 40 healthy subjects randomly selected from physical check-up department without history or clinical signs of chest pain or neurological impairment. They were matched with ACS subjects for gender and age.

Exclusion criteria included idiopathic cardiomyopathies, significant valvular heart disease, any malignancy, hematologic or rheumatologic disease, renal failure (serum creatinine ≥3), or liver diseases (elevated aminotransferases). At the end of the study, we additionally recruited 19 patients with transient ischemic attack (TIA), defined as acute stroke symptoms that recovers within 24 h of onset, for post-hoc comparison with acute LAT ischemic stroke patients.

**Plasma SCUBE1 and sCD40L assays.** Heparinized blood samples were centrifuged at 2,500 g and plasma was stored at −80°C for further analysis. High-binding, flat-bottom polypropylene 96-well plates (NUNC, Naperville, Illinois)
were coated overnight at 4°C with 50 μl of anti-SCUBE1 monoclonal Ab #712 (5 μg/ml) developed in our lab (8). The coated plates were washed (0.05% Tween-20 in phosphate-buffered saline), blocked (0.5% bovine serum albumin in phosphate-buffered saline), and incubated for 1 h with 50 μl of recombinant SCUBE1 or plasma samples in triplicate. After washing, a second horseradish peroxidase-conjugated SCUBE1 mAb #701 (5 μg/ml) was incubated for 1 h, then washed, and developed with tetramethyl benzidine (KPL, Gaithersburg, Maryland) followed by 1 N HCl. Then the absorbance at 450 nm was determined (SpectraMax 340PC, Molecular Devices, Sunnyvale, California). The minimum detection limit by this method was 50 ng/ml. The concentrations of sCD40L were determined in heparinized plasma by enzyme-linked immunosorbent assay according to manufacturer’s protocol (R&D Systems, Minneapolis, Minnesota). All assays were performed in batch by another investigator blinded to the clinical diagnoses. The intra-assay and interassay covariance of both assays was <10%.

For Group II (ACS) and III (AIS) subjects, time from onset of symptoms to blood withdrawal was recorded. To investigate the serial changes of plasma SCUBE1, we examined 8 consecutive acute STEMI patients soon after admission to coronary care units, then every 12 h for 3 to 4 days.

Characterization of plasma SCUBE1 and comparison with full-length SCUBE1. Total plasma protein was separated by 2-dimensional electrophoresis using nonlinear pH 4-7 immobilized pH gradient strips for the first dimension and 10% acrylamide gels for the second dimension. The gels were transferred to polyvinylidene fluoride membranes and stained with anti-SCUBE1 monoclonal antibody (MAb 701), followed by a secondary HRP-conjugated goat anti-mouse antibody.

To confirm that full-length SCUBE1 would be cleaved by serum proteases, we overexpressed Flag-SCUBE1 in HEK-293T cells in the presence or absence of 10% fetal bovine serum in Dulbecco’s modified Eagle’s medium. After 48 h of incubation, the medium was immunoprecipitated and blotted with anti-Flag antibody to identify the secreted Flag-tagged SCUBE1. Two carboxyl-terminal deletion mutants D1 (lacking CUB domain) and D2 (further lacking the spacer region) (1) were used as comparison.

Statistical analysis. The baseline characteristics of participants were analyzed according to case-control study design, namely CAD versus control subjects, ACS versus control subjects, and AIS versus control subjects. Demographics were presented as mean ± standard deviation for continuous variables and frequencies (percentages) for categorical variables. The chi-square test and the 2-sample t test were used to detect proportion differences and mean differences between cases and control subjects, respectively, as independent samples.

The comparisons of plasma SCUBE1 concentration between cases and control subjects and among clinical subtypes were carried out using nonparametric methods for 2 reasons: 1) lower plasma SCUBE1 concentration (<50 ng/ml) was undetectable; and 2) the distribution of plasma SCUBE1 concentration was skewed to the right, and some extreme observations remained strongly influential to the mean values. Therefore, we applied the Wilcoxon rank sum test to compare the plasma SCUBE1 concentration between cases and control subjects and Kruskal-Wallis test for comparison among clinical subtypes. To be conservative, we used 50 ng/ml to represent all values below the detection limit of 50 ng/ml for the analyses. Multiple comparisons were corrected with Bonferroni method for cases (CAD, ACS, AIS) versus control subjects (the original p values multiply by 3, at the significant level of 0.05). Similar approach was applied for the analysis of plasma sCD40L because the distribution was skewed and the lowest detection limit was 15 pg/ml. The relationship between plasma SCUBE1 and disease status as well as plasma sCD40L were further evaluated by using multiple linear regression models with log-transformed plasma SCUBE1 as outcome variable, whereas disease status, log-transformed plasma sCD40L, and all potential covariates as explanatory variables. From the full model 1, backward stepwise method with a value of p < 0.2 was applied to select important covariates to generate the simplified final model, which adequately explained the full model, confirmed by likelihood ratio test.

To explore how plasma SCUBE1 concentration was related to the severity of acute stroke, we applied multiple linear regressions with log-transformed SCUBE1 concentration as an independent variable, predicting NIHSS, assumed as a continuous variable, and adjusted for the same covariates with backward selection method. To investigate the relationship of plasma SCUBE1 concentration and onset time, we plotted plasma SCUBE1 concentration of ACS and AIS patients versus onset time, and created serial curves for 6 ACS cases. STATA (StataCorp, College Station, Texas) version 8.0 was used for all statistical calculation.

Results

Baseline characteristics of study population. Baseline characteristics of patients in the 3 cases groups versus healthy control subjects are shown in Table 1. There were no significant differences in age, gender, and platelet counts. All 3 groups had significantly higher levels of total cholesterol and higher proportions of all conventional risk factors as well as prior statin and antiplatelet use than healthy control subjects. Plasma triglyceride concentrations were significantly higher in CAD and AIS patients, and plasma creatinine levels were significantly higher in CAD and ACS patients, when compared with those of control subjects. The clinical subtypes of each group were also shown.
Plasma SCUBE1 and sCD40L concentrations in cases versus controls. The median (25%, 75%) plasma SCUBE1 concentration of subjects with CAD, ACS, and AIS were 50 (50, 50), 205 (67.6, 483.1), and 95.1 (50.0, 365.5) ng/ml, respectively. Both ACS and AIS patients had significantly higher plasma SCUBE1 than that in healthy control subjects, whose plasma SCUBE1 was mostly below detection limit (50 ng/ml), with both p values after Bonferroni correction <0.001 (Fig. 1A). Plasma SCUBE1 concentration in CAD patients was not significantly higher than control subjects. Meanwhile, plasma sCD40L concentration of CAD, ACS, and AIS patients were 28 (19, 67), 37 (20, 67), and 15 (15, 32) pg/ml, respectively. Both CAD and ACS had significantly higher plasma sCD40L than those in normal control subjects, with the level of 15 (15, 36) pg/ml.

Table 2 shows multiple linear regression models predicting plasma SCUBE1, adjusted for potential covariates. We found that both ACS and AIS were independent predictors of elevated plasma SCUBE1 concentration. Older age tends to decrease and smoking tends to increase plasma SCUBE1 concentration, as shown in final model 1. Increasing plasma sCD40L is significantly associated with increasing plasma SCUBE1, shown by unadjusted Spearman rho = 0.59 (p = 0.005) as well as the multiple regression models ($\beta = 0.32$, $p < 0.001$; $R^2 = 0.42$).

Subgroup analysis of plasma SCUBE1 and sCD40L. Figure 1B demonstrated the boxplots of both biomarkers stratified by clinical subtypes. Subjects with acute LAT stroke had significantly higher plasma SCUBE1 and sCD40L concentration compared with those in small lacunar stroke patients, both $p < 0.01$. Interestingly, LAT stroke also had higher plasma SCUBE1 than TIA patients ($p < 0.01$). Within the CAD group, the number of stenotic vessels was neither associated with plasma SCUBE1 nor with sCD40L concentration. Likewise, subjects with STEMI, NSTEMI, and UA did not differ significantly in plasma SCUBE1 and sCD40L concentration. However, these subgroup post-hoc analyses were based on small sample size, which had limited statistical power to detect differences of $<2$-fold.

Plasma SCUBE1 concentration in relationship to onset time. Figure 2A showed the scatter plot of plasma SCUBE1 concentration corresponding to the onset time in patients with ACS and LAT stroke. Plasma SCUBE1 was detectable as early as 6 h after the onset of symptoms, and not later than 84 h, both in ACS and LAT stroke. This tendency was further supported by the serial measurement of plasma SCUBE1 concentration in 6 of 8 patients with acute STEMI (Fig. 2B). Peak values occur at 36 to 60 h after onset, and degraded not later than 96 h after onset. Two other patients who received thrombolytic therapy with recombinant tissue plasminogen activator had undetectable plasma SCUBE1 for the entire serial time measurement.

Plasma SCUBE1 concentration and severity of acute thrombotic complications. Multiple regression analysis revealed that plasma SCUBE1 concentration was an independent predictor of NIHSS in LAT stroke patients, with $\beta = 3.3$ (95% confidence interval 2.77 to 3.82, $p < 0.001$; $R^2 = 0.75$) (Table 2, model 2). There was no correlation between plasma SCUBE1 and single measurement of plasma troponin I concentration in ACS patients (not shown).

Plasma SCUBE1 exists as proteolytic products of the full-length SCUBE1. To confirm and further characterize SCUBE1 in plasma, we performed 2-dimensional electrophoresis followed by Western blot on plasma samples from ACS patients with elevated SCUBE1. As shown in Figure
3A, this approach identified an acidic (pI of ~5.0) and a neutral fragment (pI of ~6.5) with molecular weight of ~95 and 80 kDa, respectively, which are different from full-length SCUBE1 protein extracted from platelets, with molecular weight of 135 kDa and the pI of 6.7 (1). It is noteworthy that the 95-kDa fragment is the reminiscent of thrombus-associated fragment described in our recent report (8).

Potential proteolytic cleavage site is located in the spacer region. As seen in Figure 3B, under serum-free conditions, the molecular weight of the secreted full-length SCUBE1 protein is ~135 kDa (1). However, in the presence of serum, a fraction of the secreted protein undergoes proteolysis by serum proteases, resulting in a cleaved fragment with the molecular weight of ~65 kDa (Fig. 3B). This SCUBE1 fragment has a molecular weight between SCUBE1 D1 and D2 mutant products, suggesting that the possible cleavage site is somewhere within the spacer region.

**Discussion**

Platelet aggregation has been well known as the culprit of ACS and AIS. Our recent study demonstrated that SCUBE1 was abundantly expressed in human platelets (8). This novel protein was predominantly stored within the platelet α-granules and only negligible amount, if any, was present on the cell surface of resting platelets. Upon platelet stimulation, SCUBE1 was translocated to platelet surface, cleaved, and then released into plasma (8). In the present study we demonstrated that plasma SCUBE1 was elevated in ACS and acute LAT stroke patients, concomitant with the increase in plasma sCD40L, suggesting platelets as the origin of plasma SCUBE1. This was further supported by the finding of 2-dimensional electrophoresis followed by Western blot, showing that plasma SCUBE1 exists as
smaller fragments, as proteolytic products of full-length SCUBE1 protein in platelets (8).

**Plasma SCUBE1: a novel biomarker of platelet activation in acute thrombotic diseases.** As shown in Figure 1, plasma SCUBE1 concentrations were significantly elevated in ACS and acute LAT stroke patients, but not in chronic CAD patients. This complies with the fact that the pathogenesis of chronic CAD is different from those of ACS. The unstable plaques in ACS consisted of large lipid core with severe inflammation, which ruptured and caused massive platelet activation. In chronic CAD, the stable plaques involved prominent smooth muscle proliferation, increased extracellular matrix synthesis that was covered by thick fibrous caps, with less inflammation and subsequently less plaque ruptures. While platelets did play a role in atherosclerosis, acute massive platelet activation was less common in chronic stable CAD (11,24). Interestingly, of seven chronic CAD patients with elevated plasma SCUBE1, we observed that 2 of them had coronary arteriovenous fistula, another 2 had relatively sluggish coronary flow, and the other 3 did not have any specific finding on coronary angiography.

In the subgroup analysis of AIS, LAT stroke but not small lacunar stroke had elevated plasma SCUBE1 concentration. Large-vessel atherothrombotic stroke involved acute platelet activation, while small lacunar stroke involved the occlusion of small penetrating artery due to lipohyalinosis from sustained hypertension, with less evidence of platelet activation (22). Our post-hoc analysis also showed that subjects with acute LAT stroke had significantly higher plasma SCUBE1 than those with TIA, indicating a potential application in the clinical setting to distinguish between

### Table 2: Multiple Regression Analyses

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<th>Explanatory Variables</th>
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<th>p Value</th>
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<td><strong>Model 1: predicting log SCUBE1 (full model)</strong> ††</td>
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<tr>
<td>ACS ‡</td>
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<tr>
<td>Stroke ‡</td>
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<td>Log sCD40L</td>
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<td>Age</td>
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*Full model 1 also included gender, diabetes, hypertension, hypercholesterolemia, platelet counts, prior antiplatelet and statin uses (all with a value of p > 0.2); †R² for full model 1; final model 1 and model 2 were 0.41, 0.42, and 0.76, respectively; ‡normal control subjects as the reference group.

ACS = acute coronary syndromes; CAD = coronary artery disease; NIHSS = National Institutes of Health stroke scale; SCUBE1 = signal peptide–complement proteins C1r/C1s, Uegf, and Bmp1–epidermal growth factor-like domain containing protein 1.

### Figure 3: Plasma SCUBE1 as Proteolytic Fragments

(A) Two-dimensional electrophoresis and Western blot of a representative plasma sample of acute coronary syndromes patients probed with antisignal peptide–complement proteins C1r/C1s, Uegf, and Bmp1–epidermal growth factor-like domain containing protein 1 (SCUBE1) antibody. Two spots (arrowheads) represent the proteolytic fragments (MW = 95 kDa, pl = 5 and MW = 80 kDa, pl = 6.5, respectively), different from the full-length SCUBE1 protein (MW = 135 kDa, pl = 6.7, arrow). These 2 fragments have been seen in multiple acute coronary syndromes plasma samples. (B) The secreted SCUBE1 protein is cleaved in the presence of fetal bovine serum (FBS). The recombinant Flag.SCUBE1 protein was produced in HEK-293T cells in the presence or absence of FBS. Immunoprecipitation of Flag.SCUBE1 was followed by Western blotting using anti-FLAG antibody. (C) Proteolytic cleavage within the spacer region of the secreted SCUBE1. Recombinant Flag.SCUBE1 full-length (FL), deletion mutants (D1 and D2) protein was compared with the cleaved SCUBE1 fragment. The positions of FL protein (arrow) or its cleaved fragments (arrowhead) are indicated. (D) Domains of the SCUBE1 full-length and deletion mutants. Arrow shows a putative protease cleavage site (RXXR) within the spacer region. “Y” indicates potential N-linked glycosylation site. CUB = complement proteins C1r/C1s, Uegf, and Bmp1 domain; E = EGF-like repeats; SP = signal peptide.
but within the spacer region (Figs. 3C and 3D). However, it revealed that the potential proteolytic cleavage site is located in-vitro study demonstrated that the secreted form of activated platelet.

Plasma SCUBE1 as proteolytic fragments derived from activated platelet. Plasma SCUBE1 in ACS patients exists as smaller fragments (Fig. 3A). Consistent with this finding, in-vitro study demonstrated that the secreted form of SCUBE1 was proteolytically cleaved by serum proteases to release the carboxyl-terminal CUB from the amino-terminal portion of the EGF-like repeats (Fig. 3B). Further mapping by using a series of SCUBE1 deletion mutants revealed that the potential proteolytic cleavage site is located within the spacer region (Figs. 3C and 3D). However, it remains to be established the precise cleavage site as well as the identity of serum protease for SCUBE1. The presence of multiple isoforms of SCUBE1 fragments suggests that the cleavage is complexly regulated in the pathological settings of ACS and AIS. The proteolytic cleavage might also represent an activation mechanism as described for the regulation of the CUB domain-containing secreted proteins, like platelet-derived growth factor-C and -D (26–28). Indeed, our recent data demonstrated that the amino-terminal 9 copies of the EGF-like repeats function as an adhesive module in mediating the platelet-matrix and platelet-platelet interactions (8). These soluble EGF-like domain fragments of SCUBE1, though alone did not trigger platelet aggregation, could potentiate ristocetin-induced platelet agglutination. Ristocetin causes conformational change of von Willebrand factor and initiates the binding of von Willebrand factor to platelet glycoprotein Ib, then induces platelets agglutination, which is a fluid-phase analogy to platelet-subendothelial matrix adhesion (29). Study limitations. While this pilot study showed a promising result of plasma SCUBE1 as a biomarker of platelet activation in acute thrombotic disease, it was based on a relatively small sample size that had limited statistical power to detect small differences in subgroup analyses. Moreover, the current enzyme-linked immunoadsorbent assay method for plasma SCUBE1 has a limited sensitivity of 50 ng/ml. Potential clinical applications. Though cardiac troponins are excellent markers for detecting myocardial infarction and can easily distinguish acute myocardial infarction from UA, however, plasma SCUBE1 as a biomarker of platelet activation, in conjunction with cardiac troponins, might further strengthen the diagnosis of UA and differentiate UA from nonspecific cause of acute chest pain. In addition, plasma SCUBE1 is also potentially useful to distinguish acute stroke and TIA as well as for risk stratification of acute LAT stroke patients. Further larger clinical studies are required to validate the clinical application of plasma SCUBE1 as a marker of platelet activation, a prognostic biomarker, as well as for monitoring response to therapy in patients with acute thrombotic complications.

Conclusions

In summary, this study is the first to demonstrate that plasma SCUBE1 concentrations are elevated in patients with ACS and acute LAT stroke. Moreover, plasma SCUBE1 concentration is an independent predictor of stroke severity as assessed by NIHSS. The soluble plasma SCUBE1 derived from stimulated platelets through a proteolytic cleavage that might play pathological roles by facilitating the platelet adhesion/agglutination and, subsequently, thrombus formation.

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Reprint requests and correspondence: Dr. Jin-Jer Chen or Dr. Ruey-Bing Yang, Institute of Biomedical Sciences, Academia Sinica, 128 Academia Road, Sec. 2, Taipei 11529, Taiwan. E-mail: jc8510@yahoo.com or rbyang@ibms.sinica.edu.tw.

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