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Original article

Early detection of C-reactive protein and von Willebrand factor levels in Malaysian patients with acute coronary syndrome

Wen Ni Tiong [a,b,*], Edmund Ui Hang Sim [b], Alan Yean Yip Fong [a,c], Tiong Kiam Ong [c]

[a] Clinical Research Centre, Sarawak General Hospital, Malaysia
[b] Faculty of Resource Science and Technology, University Malaysia Sarawak, Kota Samarahan, Malaysia
[c] Department of Cardiology, Sarawak General Hospital, Kuching, Malaysia

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ABSTRACT

Background: Diagnosing acute coronary syndrome (ACS) remains a challenge in patients presenting at early phase of hospitalization. We hypothesized that inflammatory markers of plaque rupture could accurately identifying ACS patients from stable coronary artery diseases (CAD).
Materials and methods: The serum and peripheral blood gene expression levels of C-reactive protein (CRP) and von Willebrand factor (vWF) in multiethnic Malaysian patients ($n = 7$) admitted with early hospitalization of ACS was evaluated. Nine patients with stable coronary artery disease without previous history of ACS were enrolled as controls.
Results: Serum and peripheral blood mRNA levels of CRP and vWF were significantly higher in ACS compared to control groups ($P < 0.05$).
Conclusions: Elevated levels of these markers in ACS may reflect an acute phase response due to endothelial dysfunction. Both CRP and vWF may add to the list of useful markers for early detection of ACS in hospitals of developing countries.

1. Introduction

Acute coronary syndrome (ACS) remains as one of the top causes of mortality and morbidity in both developed and developing countries. Rupture of an atherosclerotic coronary plaque i.e. the 'vulnerable plaque', is the hallmark of acute coronary syndrome.[1] Early identification of a patient during the pathophysiological process of coronary atherosclerotic plaque rupture could improve the risk stratification of patients presenting with ACS, provision of more aggressive treatment strategies to those at the highest risk, and potentially improve their clinical outcomes.

A growing number of studies reported that inflammation plays a crucial role in pathogenesis of atherosclerosis.[2–4] It was reported that the acute phase reactant C-reactive protein to be predictive of future cardiovascular events, including myocardial infarction, ischemic cardiac events or sudden death among patients with angina pectoris.[5] As a marker of systemic inflammation, it is still not known if elevated CRP levels are linked to the inflammatory response associated with endothelial dysfunction as represented by von Willebrand factor (vWF) marker in Malaysian patients with ACS. It was previously suggested that an increased level of CRP within 6 h after onset of acute myocardial infarction may signify vulnerable plaque rupture instead of a consequence of myocardial damage.[6] In addition, it had been reported that endothelial dysfunction is a critical intermediate phenotype to promote interaction between low-grade inflammation with the likelihood of thrombosis and vessel occlusion.[7] Therefore, our principal objective was to investigate the relationship between CRP and vWF, individually, and in combination, at the earliest clinical opportunity in patients presenting to hospital with ACS. By doing so, we aimed to find out if CRP and vWF can be an index for early identification of ACS among those admitted within 1 h of hospitalization after event.

2. Materials and methods

2.1. Study participants

We enrolled seven consecutive patients who were admitted to Accident and Emergency Unit of Sarawak General Hospital, Kuching. These patients had clinical characteristics consistent with ACS which associated with transient ST-segment or T-wave changes on standard 12-lead electrocardiogram or raised serum troponin T levels occurring with their typical symptom onset, were recruited

* Corresponding author. Clinical Research Centre, Sarawak General Hospital, Jalan Tun Ahmad Zaidi Adruce, Kuching, Sarawak, Malaysia. Tel.: +60 82 276 820; fax: +60 82 276 823.
E-mail address: tiongwn@crc.gov.my (W.N. Tiong).

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within 1 h of hospital admission. Nine patients with documented angiographically ≤50% coronary stenosis, who had no previous history of ACS or other acute non-cardiac vascular event were included as control. All patients provided written informed consent, and the study was approved by the Medical Research Ethics Committee of Ministry of Health Malaysia.

2.2. Measurement of serum level of CRP and vWF

Serum samples were analyzed duplicates by commercial kits of enzyme immunosorbent assay (ELISA) for high sensitivity CRP (American Diagnostica, Stamford, CT, USA) and vWF (AssayPro, St. Charles, MO, USA). The lower limit of detection by ELISA for hsCRP and vWF was 2.5 ng/ml and 5 ng/ml, respectively. The intra- and inter-assay coefficient variations for both markers were less than 8% and 10%, respectively. All final results were presented as μg/ml.

2.3. Measurement of peripheral blood MRNA levels of CRP and VWF

Comparison of gene expression levels of CRP and vWF in ACS case and control samples was via assessment of RT–PCR results between the two sample-types. Initially, total RNA for all samples were isolated from peripheral whole blood by using TRI-reagent RT-blood reagent (Molecular Research Centre Inc., Cincinnati, OH, USA) and further cleaned up using RNeasy® mini spin column kit (Qiagen, Hilden, Germany) as manufacturer's instruction. The total RNA was then reverse-transcribed to cDNA by using MMLV reverse transcriptase (Promega, Madison, WI, USA). Oligonucleotide primer sequences for CRP and vWF are shown in Table 1. All primer sequences were designed to span the intron to ensure that no false positive PCR fragments would be generated from pseudogenes.

A 25 μl PCR reaction consists of 1× Green GoTaq Flexi Buffer (Promega, Madison, WI, USA), 1.5 mM $MgCl_2$, 0.2 mMdNTPs, each of 0.5 mM forward and reverse primers, 0.03 unitTaq Polymerase and 80 ng of cDNA template. PCR reactions were run using an MJ Research DNA Engine PTC-200 Peltier Thermal Cycler (UK) as follow: Initial denaturation of 94 °C for 3 min; 40 cycles of denaturation cycles 94 °C for 30 s, annealing cycles of 59 °C (vWF) or 65 °C (CRP) for 30 s and extension cycles of 72 °C for 30 s; final extension of 72 °C for 3 min. The PCR samples were electrophoresed on 3% (w/v) agarose gels and the gel images were digitally captured with Red™ Imaging System (Alpha Innotech, San Lendro, CA, USA). The relative intensity of CRP and vWF mRNA expression was measured by densitometry (ImageJ, NIH, Bethesda, MD) and then normalized to non-targeted GAPDH mRNA expression within the same sample.

2.4. Statistical analysis

Data distribution was assessed using Shapiro–Wilk test ($n < 50$). All continuous variables had a normal distribution except for serum and mRNA levels of CRP. Thus, CRP concentrations were reported as median (interquartile range). The differences between groups were assessed using unpaired two-tailed *t*-test or Mann–Whitney test as appropriate. The differences in dichotomous variables among groups were compared with the chi-square test. Correlations were examined using Spearman's rank correlation. Multivariate linear regression analyses were used to assess the independent relationship between CRP and vWF with overall subjects after adjusting for traditional risk factors. A *P* value of ≤0.05 was considered significant. All statistical analyses were carried out using the statistical program SPSS 16.0 for Windows (SPSS, Chicago, IL, USA).

Table 1
Oligonucleotide primer sequence.

Gene	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Product size
CRP	*TGGCCAGACAGACATGTCGAGG*	*AGTGGAGGCACACAGTGAAGGC*	119 bp
vWF	*ACTTGGCCTCAACTGCCACC*	*AACGTAAGTGAAGCCCGACCG*	227 bp
GAPDH	*TGGAGAAGGCTGGGGCTCAT*	*AGTTGGTGGTGCAGGAGGCA*	153 bp

3. Results

As shown in Table 2, there was no significant difference between ACS patients and stable CAD control subjects in terms of gender, age, clinical and biochemical variables, except for systolic blood pressure and WBC which was significantly higher in ACS patients. Patients with ACS were found to have a significantly lower left ventricular ejection fraction by echocardiography compared to controls.

Both serum and gene expression levels of CRP and vWF that were measured within 1 h of hospitalization after ACS were significantly higher in ACS patients than in controls (Table 3).

Due to consideration of small sample size and the shared pathogenesis of ACS and stable CAD, we combined both ACS and stable CAD control subjects and found that WBC was correlated with both serum ($r = 0.547$, $P = 0.028$) and mRNA ($r = 0.522$, $P = 0.038$) levels of CRP; and serum vWF only ($r = 0.753$, $P = 0.001$) in overall study subjects ($N = 16$). Moreover, CRP and vWF was associated to one another, regardless of their serum ($r = 0.632$, $P = 0.009$) or mRNA levels ($r = 0.593$, $P = 0.015$).

The increased expression of CRP and vWF could be influenced by established cardiovascular risk factors. A multivariate linear regression analysis was therefore performed to assess the association of CRP and vWF with ACS subjects after adjusting for potential confounders. As shown in Table 4, the association between serum CRP and both serum and mRNA levels of vWF with ACS subjects remained highly significant ($P < 0.05$) after adjusting for the effects of cardiovascular risk factors. It was also observed that the mRNA levels of CRP could be influenced by patients with a prior history of hypertension ($r = 0.564$, $P = 0.023$).

4. Discussion

Although ACS and stable CAD are different clinical manifestations of CAD and they are associated with different prognostic outcomes, their underlying pathogenesis closely correlates with the vulnerability of an atherosclerotic plaque.[8] The only difference is correlation of ACS with erosion or a ruptured plaque while stable

Table 2
Baseline characteristics of study patients.

Patient characteristics	ACS ($n = 7$)	Control ($n = 9$)	*P* value
Male, %	71.4	88.9	0.550
Age, year	56.0 ± 7.7	53.8 ± 9.3	0.619
Clinical parameters			
BMI, kg/m^2	25.8 ± 1.5	25.7 ± 3.0	0.915
Heart rate, rpm	74.4 ± 12.4	69.6 ± 13.1	0.461
Systolic blood pressure, mmHg	158.4 ± 17.3	138.0 ± 11.9	0.014
Diastolic blood pressure, mmHg	93.7 ± 10.9	84.1 ± 3.9	0.061
Biochemical parameters			
Creatinine, mmol/L	95.7 ± 20.4	90.8 ± 11.6	0.551
Platelet count × 10^3 μl	286.7 ± 73.0	248.0 ± 28.1	0.164
WBC × 10^3 μl × 10^3 μl	9.4 ± 1.3	6.9 ± 1.1	0.001
Total cholesterol, mmol/L	5.2 ± 0.9	4.9 ± 1.1	0.581
Triglycerides, mmol/L	1.9 ± 0.9	1.9 ± 0.7	0.966
Left ventricular ejection fraction, %	41.5 ± 8.9	67.0 ± 7.6	<0.001

All differences are significant if $P < 0.05$.
WBC, white blood cells.

Table 3
Serum and mRNA levels of CRP and vWF in study patients.

Markers	ACS ($n = 7$)	Control ($n = 9$)	P value
Serum levels[a]			
CRP	6.83 (10.03)	1.06 (1.99)	0.016
vWF	15.33 ± 7.15	6.85 ± 2.09	0.004
mRNA levels[b]			
CRP	0.36 (0.47)	0.00 (0.04)	0.031
vWF	1.50 ± 0.64	0.92 ± 0.22	0.022

[a] Serum levels of both CRP and vWF were presented as μg/ml.
[b] mRNA levels of both CRP and vWF were presented as arbitrary unit, as relative to GADPH level.

CAD links to plaque stability.[9] In Malaysia, the chest pain to treatment time in the emergency department has a median time of 85 min for thrombolytic treatment[10] and these patients are typically referred more than 24 h after hospital admission for further management with a primary percutaneous coronary intervention (PCI) strategy.[11] This delay in treatment time occurred as some patients may present with atypical symptoms or showed no diagnostic changes on initial ECG tracing.[12] Currently available cardiac markers such as creatine kinase and its isoenzyme (CKMB), troponin T and troponin I are lacking of sensitivity and specificity for the initial risk evaluation as they only detect the myocardial damage phase after an ACS event has occurred.[13,14] Therefore, the search for an ideal inflammatory marker that can rapidly identify ACS at early phase of disease has taken on greater significance, especially when these are done in specialist heart centers where aggressive, even invasive, treatment strategies are available.

In this study, we demonstrated that both CRP and vWF concentrations are higher in patients with ACS and correlated with WBC in these patients. Hence, it gives further support of the two markers' role(s) in the pathogenic mechanism of CAD, other than merely markers for ACS detection.

Despite more commonly considered as an acute phase reactant, CRP is also believed to directly participate in initiation and propagation of atherosclerosis.[3] It was previously reported that the correlation between serum levels of CRP and the vulnerability of coronary plaque,[15,16] implicating that a spike in serum CRP maybe prerequisite for acute plaque rupture and erosion, which eventually leads to ACS. Meanwhile, vWF is recognized as a reliable marker of endothelial dysfunction, an early event of atherogenesis which precedes the formation of plaques.[17,18] Our findings that elevated serum levels of CRP are associated with endothelial dysfunction in atherosclerotic patients extend previous observations about inflammation and cardiovascular risk.[7,19] An enhanced release of both inflammatory markers could contribute to atherosclerosis development and progression, eventually causing myocardial damage.

The reasons to study mRNA levels are largely based on a key assumption that mRNA expression is informative in predicting protein expression level,[20] hence, it is hypothesized to be expressed earlier than the protein in relation to gene functions. Our findings on the positive correlation between serum and mRNA levels of CRP and vWF suggest that both markers could help to differentiate ACS from stable CAD patients during the early phase of hospitalization.

In this study, an increased mRNA level of CRP could be influenced by other cardiovascular risk factors, particularly in hypertensive patients. This indicates that CRP may be involved in pathogenesis of vascular injury due to subclinical inflammation.[21] Hence, the increased level in ACS may not be solely due to plaque rupture. While CRP may be useful in risk stratification of patients with ACS, interpretation of the result in patients with hypertension should be done with caution. However, our results suggest that it can be used with vWF or other plaque rupture markers to increase its detection specificity.

The present study bears several limitations. First, there is a growing concern that the levels of these inflammatory markers may fluctuate over time, which may limit their clinical usefulness in individual patients.[22] For example, the finding from Blum et al. (2009)[23] has indicated that even though in the absence of changes in health or medications, CRP levels may fluctuate over brief periods in CAD patients. Nonetheless, our ACS subjects were only recruited within a short period of time (approximately 1 h) after their hospitalization and before any pharmacotherapy was fully established. The detection of both CRP and vWF in these circumstances are highly likely to represent an acute elevation specifically for the ACS event. However, the detection of expression levels of both CRP and vWF at different time point is still needed to estimate the exact inflammatory status after an acute event, and hence may be useful to understand the underlying pathogenesis of ACS. Second, due to sample size limitation and the difficulty in determining exact time of symptom onset in study patients with respect to ascertaining similar duration from onset of ACS to blood collection among subjects, the statistical power of the data or sampling consistency could be compromised. Nevertheless, to our knowledge, this is the first report showing an independent association of both CRP and vWF with early phase of ACS in Malaysian population, where patients mostly presented late after the onset of chest pain in hospital like most developing countries. Our study also provided limited data for determining prognosis values of CRP and vWF to predict short or long term clinical outcome after ACS.

Table 4
Association of serum and mRNA levels of CRP and VWF with ACS and control subjects as dependent variables after adjustment of traditional risk factors.

	B	SE (B)	P value	Partial R^2
Serum levels				
CRP	8.88	2.75	0.014	0.774
vWF	11.28	3.87	0.022	0.741
mRNA levels				
CRP	0.43	0.31	0.203	0.469
vWF	0.72	0.27	0.033	0.708

Adjusted for age, BMI, smoking, diabetes, hypertension, hyperlipidemia and family history of CAD.
B, regression coefficient; SE (B), standard error; R^2, partial correlation coefficient after adjustment.

5. Conclusion

Our results strongly suggest that both CRP and vWF may represent biomarkers for early risk stratification of ACS to be used in hospitals of developing countries. They also suggest a possible pathophysiological link between CRP and certain cardiovascular risk factors, and indicate that plaque rupture in our ACS study subjects may be largely due to endothelial dysfunction.

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Conflicts of interest

All authors have none to declare.

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All listed authors are justifiably credited with authorship, according to the authorship criteria. Tiong WN – conduct experiments, data analysis and interpretation, drafting of the manuscript; Sim EUH – acquisition of data and critical revision of manuscript;

Fong AYY – study design, patient recruitment, data interpretation and drafting of manuscript; Ong TK – study design and critical revision of manuscript.

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