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Elevated Plasma Levels of Gas6 Are Associated with Acute Lung Injury in Patients with Severe Sepsis

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Acute lung injury (ALI) is one of the complications of severe sepsis, causing sudden deaths. However, information regarding predictive factors for the onset of ALI in severe sepsis is limited. Growth arrest-specific gene 6 (Gas6) is secreted by endothelial cells and is important for the activation of endothelium during inflammation. This study aimed to investigate the predictive effect of plasma Gas6 in patients with severe sepsis. Collection of plasma samples was carried out from 129 participants with severe sepsis following with or without ALI development. We found that the elevated levels of Gas6, interleukin-6 and -8 (IL-6 and IL-8) in plasma were associated with the ALI development (P = 0.003, 0.002, and 0.004, respectively). We also observed the robust correlation between the plasma level of Gas6 and the following ALI development to adjustment for sepsis and administration of vasopressor. Between patients with ALI (n = 18) and those without ALI (n = 111), Gas6 and the Lung Injury Prediction Score (LIPS) showed promising discrimination (AUROC, 0.74 and 0.68, respectively), and in combination with these two indexes, the AUROC was increased to 0.86 (vs. 0.74, P = 0.05), while soluble receptor for advanced glycation end products (sRAGE) and Willebrand factor (vWF) in plasma showed no predictive value for of ALI. Collectively, our findings indicate that higher levels of Gas6 in plasma are obviously correlated with ALI development. An early increase in the plasma Gas6 level suggests that endothelial injury is a key link in the pathogenesis of ALI.

Keywords: acute lung injury; Growth arrest-specific gene 6 (Gas6); interleukin-8; interleukin-6; sepsis
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Introduction

Acute lung injury (ALI) is one of the complications of sepsis known for its contribution to sudden deaths and morbidity (Fujishima et al. 2016). Although there are many ongoing clinical trials for developing new treatment strategies of ALI, limited achievements have been made in increasing the effectiveness of existing treatment methods of ALI after attack of ALI (Miyashita et al. 2016, Wang et al. 2016, Yang et al. 2016, Zhu et al. 2016). Thus, research and development of new strategies that are beneficial to the early diagnosis are essential to improve the survival of ALI patients.

To distinguish patients with high risks of ALI development, a variety of clinical prediction scores have been developed, including lung injury prediction score (LIPS) (Gajic et al. 2011, Kor et al. 2011, Trillo-Alvarez et al. 2011). Despite that the superiority in negative predictive value (NPV; 0.96-0.98 at LIPS cut points between 4 and 6 points) of LIPS has been confirmed in some studies, its positive predictive value (PPV) ranged from 0.14 to 0.23) shows a poor value in prediction (Gajic et al. 2011, Kor et al. 2011, Trillo-Alvarez et al. 2011). In order to further improve the clinical prediction scores, researchers prefer to combine clinical data with plasma biomarkers that can be used for measuring their corresponding aspect of ALI. In fact, researchers have already applied this method in mortality prediction among ALI patients, and obtained more information through biomarkers than clinical information alone (Ware et al. 2010, Calfee et al. 2011).

Growth arrest-specific gene 6, also known as Gas6, was firstly mentioned in fibroblasts under growth arrest (Li et al. 2017, Nassar et al. 2017) and serves as the coding gene of Gas6 protein, a vitamin K-dependent protein that is principally expressed in endothelial cells, etc. (Happonen and Dahlback 2016, Jung et al. 2016, Lee et al. 2016). Gas6 is associated with cardiovascular disease (CVD) and provides further evidence that the AA genotype of the

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c.834+7G>A Gas6 polymorphism may have a protective role against acute coronary syndrome (ACS) (Jiang et al. 2009). In addition, elevated levels of Gas6 protein are associated with a variety of disease states, including venous thromboembolic disease, systemic lupus erythematosus, chronic renal failure, and preeclampsia (Dihingia et al. 2017). One recent study also suggested that the level of Gas6 in plasma serves as a mortality predictor in patients with sepsis (Stalder et al. 2016). However, it is unknown whether Gas6 is a useful marker to predict the subsequent progression of ALI in patients with severe sepsis.

In this study, we tested the performance of plasma levels of Gas6, interleukin-6 (IL-6), IL-8, Willebrand factor (vWF), and soluble receptor for advanced glycation end products (sRAGE) at admission of patients, some of which had been proved to be associated with the pathogenesis and clinical outcome of ALI (Fremont et al. 2010, Barnett and Ware 2011, Nakstad et al. 2016, Pfeiffer et al. 2017). On the basis of our results, we postulated that earlier detection plasma level of Gas6 in patients with severe sepsis is predictive to the subsequent progression of ALI, and is supplementary to the LIPS.

Methods

Subjects

A total of 162 critically ill patients who were admitted to the intensive care unit in our hospital from the ER were enrolled in this study. Patients were diagnosed as sever sepsis if they were delivered from the ER to an ICU at the request of emergency physician. However, patients who were admitted to this hospital and diagnosed as neurosurgical diseases but with no complications, or for trauma service were excluded from this study. This study had been approved by Changhua Show-Chwan Memorial Hospital and the written informed consents were obtained from all participants.

Primary outcome and other variables

Immediately after patients were delivered to the ER, we collected the plasma samples of patients, but patients whose samples were obtained over 24 hours after being delivered to ICU were excluded. Two physicians unaware of results of enzyme-linked immunosorbent assay (ELISA) were designated to adjudicate the primary outcome, wherein ALI was developed at least 6 h after collection of plasma samples. Thereafter, a 2-week follow-up was carried out for all patients to confirm the diagnosis of ALI and a 60-day follow-up to evaluate the mortality. The accurate time of ALI onset was defined as previous report (Bernard et al. 1994). To ensure ALI removal present at baseline, patients (n = 21) satisfying the criteria of ALI initially before collection of plasma samples, or within the following 6 hours were excluded from this study. In addition, we excluded 19 patients whose chest radiograph showed bilateral infiltrates but with no arterial blood gas in 24 hours after chest radiograph for we could not adjudicate the clinical outcome with the imperfect clinical data.

ELISA for measuring the protein levels of Gas6, IL-6, IL-8, vWF and sRAGE rotein in plasma

The levels of Gas6, sRAGE, IL-6, IL-8 and vWF in plasma were measured via ELISA method in accordance with the corresponding instructions (R&D, USA). Briefly, 50 μL standards and samples were evenly spread on the microtiter plates, and in each well, enzyme conjugated reagent (100 μL) was added followed by mixing for 15 s; after incubation at 36°C for 1 hour, plate was rinsed 5 times using washing buffer, and 50 μL of reagent A and B was added to each well followed by mixing for 15 s and incubation at 36°C for 15 min. Reaction was terminated through adding 50 μL stop solution, and the plate was gently mixed until the blue color turned into yellow. Optical density (OD) was measured at wavelength of 450 nm using a microplate reader in 30 min after the stop solution was added. The coefficients of variation (CV) in intra- and inter-assay were respectively 6.3% and 8.7%.

Statistical methods

In addition to the discrimination, sensitivity and specificity of biomarkers at the cut point with a minimal distance to perfect sensitivity and specificity were also calculated. In this study, bootstrap method was utilized to acquire the confidence intervals (CIs) for AUROC. Between models, we compared the discrimination and improvement in discrimination in accordance with the previously described method (Pencina et al. 2008). For discrimination of models, 10-fold cross-validation technique was adopted.

By establishing multivariate logistic regression models, we adjusted for potential confounding (severity of sepsis and other risk factors associated with infection), and clarified the additional predictive value of a biomarker in comparison with biomarkers of illness severity. To adjust for severe sepsis and score of APACHE II, an *a priori* multivariate model was prepared. Prior to inclusion of biomarkers, a natural log transformation was carried out, so as to better conform to the assumption of linearity. Hosmer-Lemeshow (HL) test was performed for checking all models. $P < 0.05$ suggested that difference was statistically significant. Data were analyzed with STATA/IC 12.

Results

Clinical characteristics of participants

The baseline characteristics of samples are shown in Table 1. It was shown that compared with patients without ALI, those with ALI caused by severe sepsis were in severe condition, with a higher score of APACHE II and dose of vasopressor, and the possibility of intubation in ALI patients than those without ALI. Among the excluded patients for a history of ALI prior to the diagnosis with their samples, they were similar to those included ALI patients in severity of condition and the pattern of distribution of ALI-associated risk factors.

We collected the plasma samples after patients were delivered into the ER (range of 0.2 to 33 hours with a median of 9.9 hours), and before and after admission to ICU (range of −14 to 24 hours with a median of 2.5 hours). For patients with attack of ALI within at least 6 hours after sample collection, the duration from the collection of plasma sample to the onset of ALI was within 6 to 100 hours with a median of 22 hours and a single outlier of 185 hours. The patient with a single median of 185 hours was excluded from this study, and the following sensitivity analysis revealed no changes in results presented below.

Table 1. Baseline characteristics.

	No ALI (n = 111)	Develop ALI (n = 18)	P Value
Age, yr	64 ± 17	65 ± 18	0.42
Male sex	60 (54)	10 (56)	0.7
Race			
Asian	111 (100)	18 (100)	
Admitting service			0.8
Medicine	88 (79)	15 (83)	
Cardiology	11 (10)	1 (6)	
Surgery	8 (7)	0 (0)	
Other	6 (5)	3 (17)	
Primary admission diagnosis			0.005
Cardiac	13 (12)	4 (22)	
Respiratory	20 (18)	3 (17)	
Gastrointestinal	33 (30)	4 (22)	
Infectious	35 (32)	3 (17)	
Neurological	9 (8)	0 (0)	
Other	1 (1)	4 (22)	
Primary ALI risk factor			0.6
None	0 (0)	0 (0)	
Sepsis	111 (100)	18 (100)	
Pneumonia	46 (41)	9 (50)	
Transfusion	18 (16)	2 (11)	
Aspiration	40 (36)	5 (27)	
Pancreatitis	6 (5)	2 (11)	
Drug overdose	1 (1)	0 (0)	
Other	0 (0)	0 (0)	
APACHE II score	25.5 ± 5.5	30.5 ± 7.5	0.003
Vasopressor use in ED	32 (29)	11 (61)	0.01
Intubated during stay	65 (59)	15 (83)	0.003
60-d mortality	31 (28)	8 (44)	0.45

ALI, Acute Lung Injury; Gas6, Growth arrest-specific gene 6; APACHE, Acute Physiology and Chronic Health Evaluation; ED, emergency department. Data are presented as mean ± SD or n (%). Analysis performed using Wilcoxon rank-sum, chi-square test, or Fisher exact test as appropriate.

Predicating the development of subsequent ALI with plasma biomarkers

Compared with patients without ALI development, the levels of Gas6, IL-6 and IL-8 were significantly elevated among patients with ALI ($P < 0.01$; Fig. 1A-C). However, there was no statistically significant difference in comparison of sRAGE ($P > 0.05$) and vWF ($P > 0.05$) levels in plasma among different groups (Fig. 1D, E). No further analyses of sRAGE and vWF were conducted for their poor predictive values for ALI.

Adjustment for multivariate in prediction model

The logistic regression model revealed that the single application of Gas6 could predict the onset of ALI (Table 2). Also, ALI could be predicted by IL-6 (OR: 1.3; 95% CI: 1.2-2.1) and IL-8 (OR: 1.4; 95% CI: 1.1-2.2).

In this study, although patients were diagnosed as sepsis, their degree of condition differed from each other. Thus, sepsis severity was considered to be firstly adjusted. Despite of the adjustment among different models, significant correlation was found between the level of Gas6 in plasma and ALI (Table 2), and, similarly, the correlation between infection-associated risk factor of ALI and ALI remained robust to adjustment (Table 2).

Adjustment was also made for biomarkers indicating the severity of illness in multivariate model, so as to guarantee the additional predictive value of Gas6 except of the ability in identifying patients with possibility to develop ALI (Table 2), and the results indicated that Gas6 was significantly associated with the onset of ALI, which was still robust to adjustment for administration of vasopressor. Even though data in the late stage (within 24 hours after

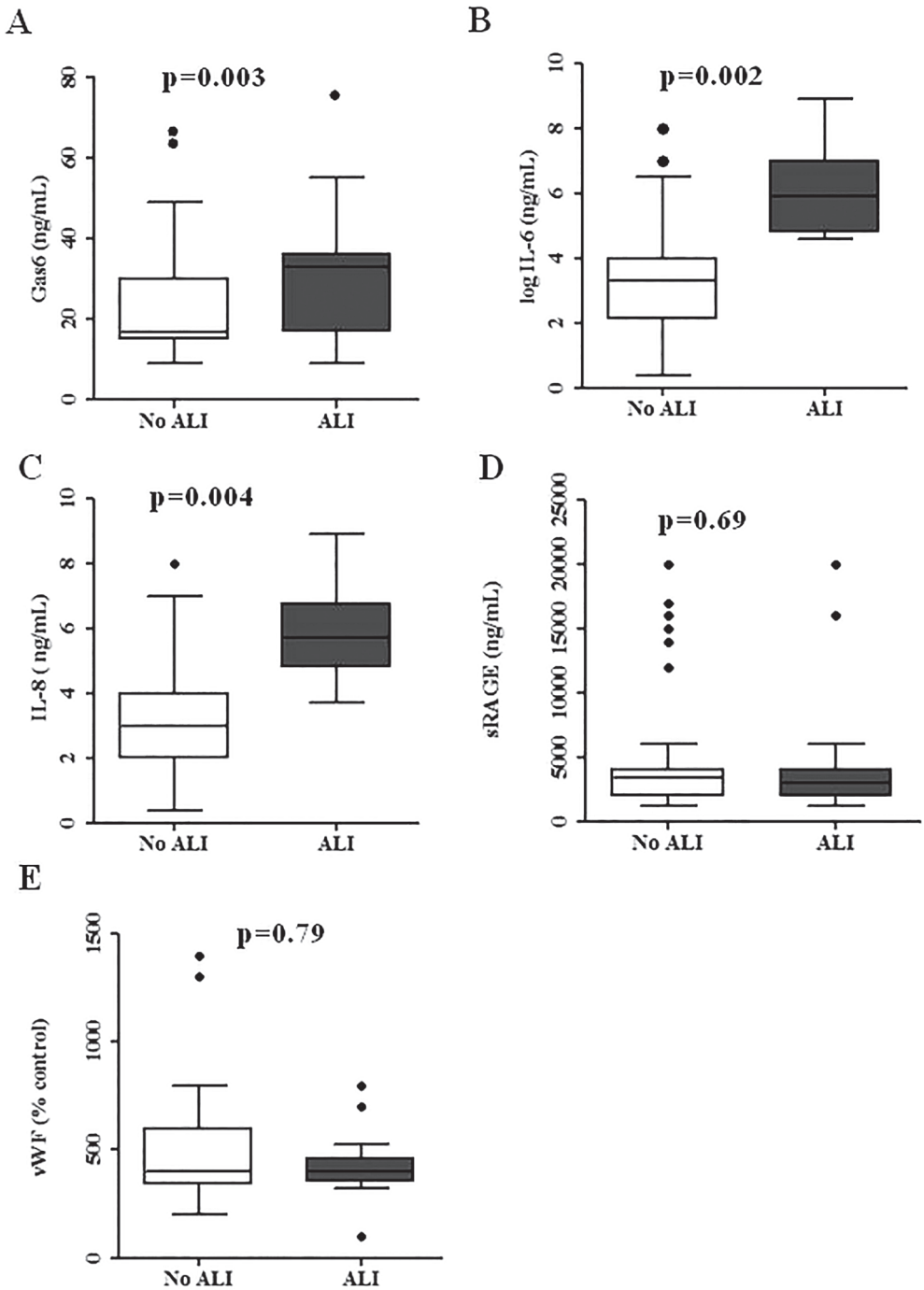

Fig. 1. Levels of biomarkers in plasma in patients with ALI. In comparison with patients without ALI (n = 111), the levels of Gas6 (A), IL-6 (B) and IL-8 (C) are significantly increased in plasma of those with ALI developed within 6 hours after sample collection (n = 18), and the differences were statistical significance. Comparisons of sRAGE (D) and vWF (E) showed no difference.

ICU admission) of patients with critical condition were integrated into the APACHE II score, mild attenuation was identified in correlation between Gas6 and ALI after adjustment of APACHE II score as well. Besides, mild attenuation was only found in the model after adjustments for APACHE II score and severe sepsis on D1.

The level of IL-6 (OR: 1.5; 95% CI: 1.3-2.0) or IL-8 (OR: 1.4; 95% CI: 1.1-2.0) was obviously correlated with ALI alone or after adjustment for administration of vasopressor in ER. Nevertheless, less robustness was manifested in correlations of IL-6 (OR: 1.4; 95% CI: 0.96-1.7) and IL-8 (OR: 1.2; 95% CI: 0.95-1.9) to adjustment for severe sepsis. In addition, similar robustness was also seen after adjustment for APACHE II score (IL-6: OR: 1.4, 95% CI: 0.98-1.7; IL-8: OR: 1.4, 95% CI: 0.92-1.7) and ALI risk factor was associated with infection (IL-6: OR: 1.5, 95%

Table 2. The association between Gas6 and prediction of acute lung injury in multivariate analyses.

Model	OR	95% CI	P Value
Gas6 + sepsis Day1	2.2	1.1-4.9	< 0.01
Gas6 + severe sepsis Day1	2.2	1.2-3.0	< 0.01
Gas6 + ED vasopressors	2.4	1.3-2.5	0.01
Gas6 + APACHE II	1.6	1.3-2.6	0.06
Gas6 + infection-related ALI risk	2.3	1.2-3.7	0.02
Gas6 + APACHE II + sepsis Day1	1.6	1.2-4.4	0.06

ALI, acute lung injury; Gas6, Growth arrest-specific gene 6; APACHE, Acute Physiology and Chronic Health Evaluation; ED, emergency department; OR, odds ratio. Infection-related ALI risk includes all patients with sepsis or pneumonia as a risk factor for ALI. Gas6 is natural log-transformed in the logistic regression model to meet assumption of linearity with log-odds of outcome. The ORs presented here are for each log increase in the level of plasma Gas6.

CI: 0.88-1.9; IL-8: OR: 1.4, 95% CI: 0.93-1.8).

Discrimination in predictive models of biomarker

In assessment of discrimination, calculation of AUROC was carried out. Results showed that good discrimination was manifested in independent prediction of Gas6 (Fig. 2) as well as LIPS score (Fig. 2) between patients who would possibly develop ALI and those who would never develop ALI. Combined model was established by integrating Gas6 into LIPS score showed that the discrimination was more significant in comparison with single application of LIPS ($P < 0.05$; Fig. 2), suggesting that LIPS model can be improved by supplementation of Gas6 ($P < 0.05$).

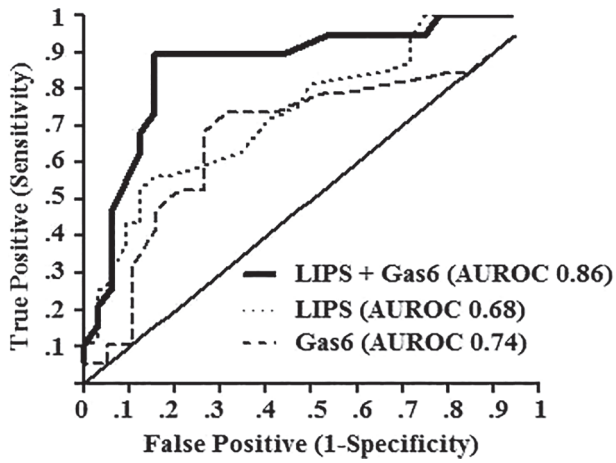

Fig. 2. ROC curves. Predictive value of Gas6 and LIPS score alone exhibit a promising discrimination between patients with ALI (n = 18) and those without ALI (n = 111), while LIPS score combined with Gas6 was superior to sole application of either of them (AUROC: 0.86; $P < 0.05$).

Features of Gas6 and LIPS in ALI prediction

Based on the conventional calculation of AUC, Gas6 cut point was set as 18 ng/mL, where the sensitivity, specificity, PPV and NPV of Gas6 for ALI were respectively 78%, 72%, 21% and 95%. However, PPV could be as high as 35% (n = 17) in predicting the development of ALI using LIPS (LIPS > 4) in combination with Gas6 (Gas6 ≥ 18 ng/mL) with substantial increases of exceeding 20 to 25% of remaining variables alone. On the contrary, with the cut points above, combination of LIPS and Gas6 in predicting the development of ALI showed that the NPV was 100% (n = 72).

Assessing the risk of ALI via sensitivity analysis in a restricted population

Among 103 patients with a risk factor of ALI development at the beginning of this study, 17 of which were identified with ALI at least 6 hours after sample collection, we performed the sensitivity analysis. Compared with patients with no ALI development, Gas6 level remained higher than those with ALI development, and the difference had statistical significance ($P < 0.05$). Also, in this subset, single application of Gas6 or LIPS had a significant discrimination in prediction of ALI development, and similarly, integration of Gas6 into LIPS score (AUROC: 0.75; 95% CI: 0.68-0.81) increased the discrimination in comparison with single application of LIPS. In sensitive analysis, ORs or P values were hardly changed due to the restriction in population at risk of ALI development in the multivariate models.

Evaluating the correlation of Gas6 with ALI in sensitivity analysis

For discovering the role of Gas6 in early stage of ALI development, the levels of Gas6 in plasma were compared

between patients with ALI and those with no ALI, and the results, similar to our previous results, showed that Gas6 was remarkably higher in ALI patients than those without ALI.

Discussion

Nowadays, prophylaxis and early treatment of ALI have attracted the attention of many researchers (Levitt and Matthay 2012), which has rendered the high priority to development of effective treatment methods. In this study, among patients who were diagnosed as severe sepsis, we found that elevation in Gas6 level in plasma could predict the development of ALI despite of the corresponding adjustment for confounding factors like severe sepsis; besides, it could also improve the parameters of the clinical prediction score, such as discrimination, PPV and NPV. In addition, we postulated that vWF and sRAGE could be used for prediction of ALI and improving the LIPS score, and the results further confirmed that these two biomarkers exhibited good performance in prediction. It had been previously reported that these two biomarkers were correlated with the prediction in ALI development, where in vWF could be used to predict the development of ALI among non-pulmonary sepsis patients (Rubin et al. 1990), while sRAGE served as an indicator in prediction of ALI development in pediatric patients who received the cardiac surgery (Liu et al. 2012). However, totally different from those results, our data indicated that sRAGE and vWF had no predictive value for development of ALI. Hence, based on the results of this study, it is highly recommended that the Gas6 level in plasma in first 24 hours after patients were delivered into the ICU may be beneficial to prediction of ALI development in sepsis patients.

Many researchers are focusing on the application of clinical prediction scores appropriate for prediction in patients at a specific risk, like LIPS (Kor et al. 2011) or post-trauma prediction model (Rainer et al. 1999), which, however, have not been verified previously, and may not be applicable to all patients with a risk of developing ALI, particularly those with conditions like severe sepsis or septic shock. A great number of studies have verified the availability of LIPS in a large population, and LIPS is excellent in prediction of ALI due to its NPV (Gajic et al. 2011, Trillo-Alvarez et al. 2011). It is rationally postulated that there would be biomarkers in plasma that are beneficial to improving the LIPS score, since integrating these biomarkers into LIPS score is conducive to increasing the value of prognostic scores in prediction of ALI-caused morality (Ware et al. 2010, Calfee et al. 2011). In this study, integration of cut point of Gas6 level in plasma into that of LIPS contributed to a significant improvement in PPV, which was superior to the prediction of ALI using LIPS score alone. In spite of the low PPV (38%) in this study, results above are still suggestive to setting the criteria for following clinical trial, since LIPS has been used to expand the ALI populations. Moreover, integration of the Gas6 cut point perfected the NPV, suggesting that LIPS in combination with Gas6 is of great significance for excluding the risk of ALI development.

In models without any adjustment, levels of IL-6 and IL-8 manifested the predictive value in predicting the development of ALI and increasing the discrimination of LIPS that were similar to Gas6; however, after adjustment in multivariable models, the predictive value of IL-6 and IL-8 was attenuated. Besides, to discover the predictive roles of IL-6 and IL-8, we need to carry out more cohort studies. These results are extremely important as they have provided us with potential methods for predicting the development of ALI and a promising perspective for exploring the pathogenesis of ALI in an early stage. Superior to other biomarkers, like sRAGE and vWF, Gas6, with the peculiar and newly-discovered ability, can deepen the understanding on the pathogenesis of ALI in an early stage. Literatures have reported the credible effect of Gas6 as a biomarker in diagnosis of acute coronary syndrome (ACS) (Jiang et al. 2009), and the association between the Gas6 and various diseases, such as sepsis (Ekman et al. 2010). The results of this study showed that Gas6 could be used to predict the development of ALI, suggesting that innate immune response is the first mechanism in response to injuries to patients at an early-stage ALI (Nassar et al. 2017).

Our study has some limitations. Owing the relatively small number of patients, these results require further validation in a larger and multi-centric cohort of patients with sepsis, which could, furthermore, allow extensive model making.

In summary, this study discovered that a combined model integrating the biomarkers, like Gas6, showed a promising prospect in developing a new method for clinical stratification of patients in an effective way, which will benefit the future clinical trials for developing new strategies in treatment and prophylaxis of ALI. Furthermore, the increase in Gas6 in an early stage suggested the significance of innate immune response in response to ALI, and attests the potential of Gas6 as a target in ALI.

Conflict of Interest

The authors declare no conflict of interest.

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