

Clinical Significance of Serum Kallistatin and ENOX1 Levels in Patients with Coronary Heart Disease

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Significance of the Study

- Kallistatin and ENOX1 may participate in the regulation of oxidative stress and are thus potentially associated with atherosclerosis.
- This study showed that levels of kallistatin were decreased in patients with coronary heart disease (CHD), while levels of ENOX1 were increased in patients with acute coronary syndrome.
- Low levels of kallistatin or high levels of ENOX1 may lead to high Gensini score in CHD. Kallistatin may be of value in the diagnosis of CHD.

Keywords

Kallistatin · ENOX1 · Gensini · Biomarker · Coronary heart disease

Abstract

Background: Kallistatin and ENOX1 are regulators of inflammation and oxidative stress which are typical pathological reactions in atherosclerosis. However, there is limited information of kallistatin and ENOX1 in coronary heart disease (CHD). **Methods:** Fifty healthy controls, 56 stable angina pectoris (SAP) patients, and 47 acute coronary syndrome (ACS) patients were included in this study. Levels of kallistatin and ENOX1 in serum were measured by ELISA. χ^2 test was performed to analyze categorical data. ANOVA, Pearson correlation analysis, and multiple linear regression were performed to analyze the numerical data. Finally, receiver operating characteristic (ROC) curve was applied to assess the diagnostic value of kallistatin in CHD. **Results:** Among the 153 par-

ticipants, 59.5% were male and the average age was 63.8 ± 11.39 years. Compared with the control group, kallistatin expression was decreased in the SAP and ACS groups while expression of ENOX1 was increased in the ACS group ($p < 0.05$). Pearson correlation analysis showed that the kallistatin level was negatively correlated with the Gensini score ($r = -0.210, p < 0.01$), white blood cell (WBC) count ($r = -0.283, p < 0.001$), and triglyceride levels ($r = -0.242, p < 0.01$) and positively correlated with age ($r = 0.353, p < 0.001$) and high-density lipoprotein cholesterol ($r = 0.310, p < 0.001$). ENOX1 expression was positively correlated with WBC count ($r = 0.244, p < 0.01$), international normalized ratio ($r = 0.177, p < 0.05$), and Gensini score ($r = 0.201, p < 0.05$). Multiple linear regression showed that Cr, alanine transaminase, glucose, and kallistatin are independent predictors for Gensini score. The ROC curve showed that kallistatin had the highest diagnostic significance ($p = 0.007$) when the area under curve was 0.636, with a sensitivity of 0.735 and a specificity of 0.495. **Conclusion:** Expression of kallistatin was decreased in

CHD patients and that of ENOX1 was increased in ACS patients. Kallistatin and ENOX1 were closely connected with the severity of CHD, and kallistatin may be helpful in the diagnosis of CHD.

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Introduction

Coronary heart disease (CHD) is one of the world's most common diseases with high fatality and disability rates [1]. CHD mainly consists of stable angina pectoris (SAP) and acute coronary syndrome (ACS) which is subcategorized into ST-elevation myocardial infarction, non-ST-elevation myocardial ischemia, and unstable angina [2, 3]. Atherosclerosis is the most common pathological mechanism of CHD [4]. According to previous studies, activation of inflammatory response, the release of cytokines and chemokines, and enhancement of oxidative stress are all involved in atherosclerosis formation. Oxidative stress has been considered to be a core factor in the pathogenesis of atherosclerosis [5].

Kallistatin is a kallikrein-binding protein and widely distributed in various tissues and body fluids. As an endogenous serine proteinase inhibitor (serpin), kallistatin plays a vital role in a variety of diseases through regulation of inflammation, oxidative stress, apoptosis, and angiogenesis [6–8]. NADH oxidase, a plasma membrane electron transport complex, is a crucial source of reactive oxygen species and a key element in the activation of oxidative stress [9]. ENOX1 is a highly conserved NADH oxidase that oxidizes NADH to NAD⁺ [10]. Kallistatin was previously reported to inhibit superoxide formation and NADH oxidase activity [11]. Therefore, there may exist a biological connection between kallistatin and ENOX1.

Even though oxidative stress has been proven to predict the risk of cardiovascular events of CHD patients decades ago [12], the clinical roles of kallistatin and ENOX1 in CHD are yet to be explored. In this study, we examined the expression profile of kallistatin and ENOX1 in the serum of CHD patients and tried to explore the relationship between these 2 factors and CHD.

Methods

Study Population

The study was approved by the Ethics Committee of Southeast University affiliated to Zhongda Hospital, and all participants provided informed written consent before they were included in the

study. A total of 153 patients hospitalized in the cardiovascular department from January 1 to March 28, 2017 were enrolled in our study. Patients suffering from more than one of the following diseases were excluded: chronic heart failure (grade III–IV), myocarditis, cardiomyopathy, pericardial disease, moderate to severe valve disease, severe hepatic or renal disease, thyroid disease, severe infection, systemic autoimmune diseases, and malignancy. All subjects were examined by coronary angiography to determine the extent of coronary artery lesions. Forty-seven patients of the enrolled patients were assigned to ACS group, which included unstable angina, non-ST-elevation myocardial ischemia, and ST-elevation myocardial infarction. The specific definition of ACS has been previously reported [13]. Fifty-six patients who had at least one major branch stenosis over 50% without any indications of ACS were classified into the SAP group. The remaining 50 patients who had normal coronary arteries were assigned to the control group.

Baseline Information Collection

Baseline characteristics of subjects, including age, sex, history of hypertension, hyperlipidemia, diabetes, smoking, and drinking as well as family history of CHD were noted. The following parameters were studied: leukocyte count, red blood cell count, platelet count, creatine kinase-MB, myoglobin, international normalized ratio (INR), prothrombin time (PT), activated partial thromboplastin time (APTT), D-dimer, triglyceride (TG), total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol (HDL-C), apolipoprotein A1, apolipoprotein B, lipoprotein (a) (lipoa), blood glucose, and Cr were collected. The left ventricular ejection fraction (LVEF), interventricular septum, left ventricular (LV), left ventricular posterior wall (LVPW), and left atrial (LA) were evaluated by echocardiography.

Measurements of Kallistatin and ENOX1

Blood samples from all the participants were collected prior to coronary angiography and stored in vacuum blood collection tubes containing inert separating gum for 15 min before serum separation. Blood samples were centrifuged at 1,000 g for 10 min at 4°C, and serum was collected and cryopreserved at –80°C until testing. The levels of kallistatin and ENOX1 were detected by an ELISA kit (Shanghai Westang Bio-tech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The highest and lowest points of the standard curve for kallistatin were 8,000 and 125, while for ENOX1 were 2,000 and 31.2.

Coronary Angiography

Coronary angiography was performed using the standard Judkins' technique. The analysis of angiographic results was performed by 2 experienced interventional cardiologists who did not know any clinical information of these patients. Gensini score was used to assess the severity of CHD. The scoring method has been previously described [14], and the higher the score, the worse the condition of coronary heart disease (CHD).

Statistical Analysis

Data processing was carried out with SPSS 22.0 and GraphPad prism 7. Continuous parametric variables were presented as mean ± standard deviation (mean ± SD). χ^2 test was performed for the categorical data. Parametric variables in groups were compared by ANOVA. The connection between variables with kallistatin and

Table 1. Basic characteristics of the subjects

	Control (n = 50)	SAP (n = 56)	ACS (n = 47)	p value
Age, years	62.26±11.36	63.39±9.95	65.94±12.86	0.269
Male, n, %	19, 38	34, 60.7	38, 80.9	<0.001
Medical history, n, %				
AF	2, 4	7, 12.5	5, 10.6	0.29
Smoking	9, 18	17, 30.4	32, 68.1	<0.001
Diabetes	6, 12	16, 28.6	14, 29.8	0.049
Hypertension	24, 48	40, 71.4	28, 59.6	<0.01
PCI History	0, 0	16, 28.6	4, 8.5	<0.001
Biochemistry detection				
RBC, ×10 ¹² /L	4.57±0.54	4.55±0.52	4.505±0.6	0.827
WBC, ×10 ⁹ /L	6.44±2.08	6.6±1.66	10.05±3.9 ^a	<0.001
Neutrophil, ×10 ⁹ /L	4.41±1.97	4.34±1.74	7.91±3.1 ^a	<0.001
Monocyte, ×10 ⁹ /L	0.35±0.11	0.39±0.15	0.45±0.16 ^a	0.003
Lymphocyte, ×10 ⁹ /L	1.55±0.58	1.74±0.63	1.55±0.93	0.315
Basophil, ×10 ⁹ /L	0.02±0.01	0.02±0.01	0.03±0.05	0.304
Eosinophil, ×10 ⁹ /L	0.11±0.12	0.1±0.08	0.13±0.19	0.691
PT, S	11.42±0.69	11.25±1.72	15.39±12.28 ^b	0.005
APTT, S	36.27±37.33	32.07±13.65	49.5±40.77 ^b	0.023
INR	1.06±0.06	1.22±1.37	1.41±1.08	0.247
D-dimer, µg/L	103.72±121.53	103.71±143.52	178.19±202.31	0.031
TC, mmol/L	4.59±1.04	4.22±1.13	4.43±0.81	0.207
TG, mmol/L	1.55±1.11	1.79±2.47	2.12±1.99	0.392
LDL-C, mmol/L	2.72±0.81	2.49±0.84	2.8±0.61	0.121
HDL-C, mmol/L	1.25±0.27	1.13±0.26	0.98±0.16	<0.001
ApoA1, g/L	1.13±0.22	2.57±11.09	0.88±0.19	0.425
ApoB, g/L	0.82±0.21	0.78±0.21	0.84±0.17	0.07
Lipoa, mg/L	318.85±271.95	327.52±372.52	339.09±251.2	0.955
Total protein, g/L	65.61±12	64.75±5.71	61.3±5.92 ^b	0.032
Albumin, g/L	41.18±7.67	41.65±3.98	37.62±4.43 ^a	0.001
Prealbumin, g/L	0.21±0.06	0.21±0.05	0.16±0.06	0.000
Cr, g/L	71.46±17.54	79.86±24.58	95.51±33.33 ^a	<0.001
BUN, mmol/L	5.22±1.31	5.78±1.4	6.78±2.74 ^a	<0.001
ALT, U/L	23.62±15.05	26.72±27.09	42.89±23.25 ^a	<0.001
AST, U/L	22.13±9.19	23.5±12.81	149.15±148.78 ^a	<0.001
Tbil, µmol/L	11.59±6.3	11.73±9.93	15.4±11.21	0.093
Dbil, µmol/L	2.66±1.98	2.46±0.99	4.95±8.26 ^b	0.024
Uric acid, µmol/L	319.23±95.62	312.03±109.71	373.28±100.74 ^b	0.007
Glucose, mg/L	5.59±1.07 ^c	7.07±3.23	7.94±4.14	0.001
Echocardiography index				
LVEF, %	0.67±0.06	0.64±0.11	0.61±0.12 ^b	0.039
IVS, cm	0.98±0.14	1.03±0.23	1.08±0.13	0.057
LV, cm	4.55±0.39	4.68±0.51	4.83±0.6	0.089
LVPW, cm	0.93±0.12	0.99±0.15	1.07±0.14 ^a	<0.001
LA, cm	3.66±0.44	3.91±0.62	3.97±0.48	0.024

Data expressed as mean ± SD or n (%). SAP, stable angina pectoris; ACS, acute coronary syndrome; AF, atrial fibrillation; WBC, white blood cell; PT, prothrombin time; APTT, activated partial thromboplastin time; INR, international standardized ratio; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; lipoa, lipoprotein a; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Tbil, total bilirubin; Dbil, direct bilirubin; LVEF, left ventricular ejection fraction; IVS, interventricular septal; LV, left ventricle; LVPW, left ventricular posterior wall; LA, left atrial. ^a $p < 0.001$ in comparison with the control and SAP group. ^b $p < 0.05$ in comparison with the control and SAP group. ^c $p < 0.05$ in comparison with the SAP and ACS group.

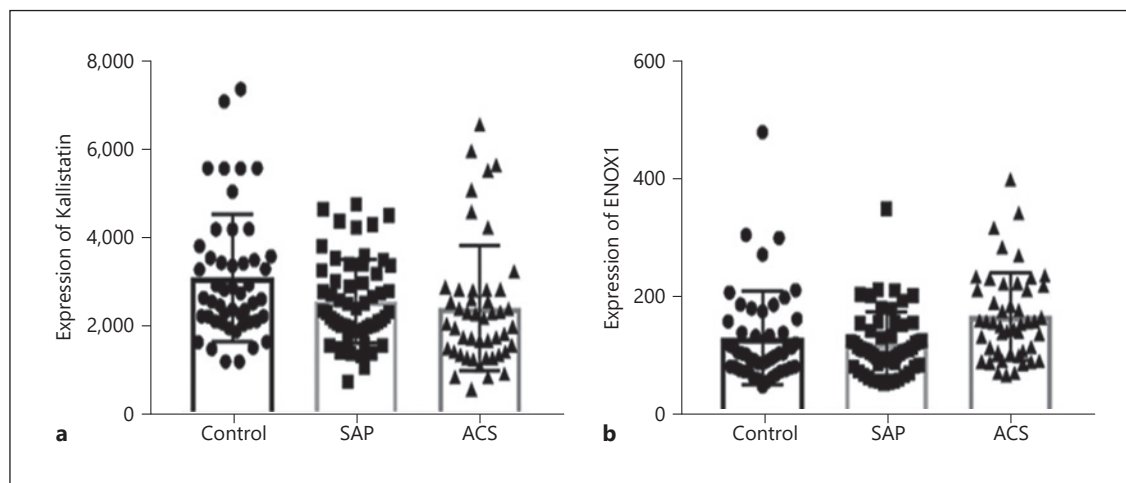


Fig. 1. ELISA levels of kallistatin (a) and ENOX1 (b) in controls, SAP, and ACS. SAP, stable angina pectoris; ACS, acute coronary syndrome.

Table 2. Comparisons of serum levels of kallistatin and ENOX1 in patients with CHD and control group

	Control (n = 50)	SAP (n = 56)	ACS (n = 47)	p value
Kallistatin, pg/mL	3,094.69±1,435.65 ^a	2,564.15±955.32	2,411.85±1,427.91	0.024
ENOX1, pg/mL	129.23±79.43	119.07±53.98	166.53±74.49 ^b	0.002
Gensini score	1.44±2.91	17.41±19.16 ^c	58.3±41.24 ^d	<0.001

Data expressed as mean ± SD or n (%). CHD, coronary heart disease; SAP, stable angina pectoris; ACS, acute coronary syndrome. ^a $p < 0.05$ in comparison with the control and SAP group. ^b $p < 0.05$ in comparison with the control and SAP group. ^c $p < 0.001$ in comparison with the control group. ^d $p < 0.001$ in comparison with the control and SAP group.

ENOX1 was assessed using Pearson correlation analysis. Multiple linear regression analysis was used to determine the correlation between kallistatin and Gensini score with related factors. The specificity, sensitivity, and area under the curve of the receiver operating characteristic (ROC) curve were also computed to assess whether kallistatin could act as a biomarker for CHD patients. $p < 0.05$ was considered to be significant for all tests.

Results

Baseline Characteristics of Patients

The general data of the study population are shown in Table 1. Among the 153 participants, there was no significant difference in age and atrial fibrillation history in the control, SAP, and ACS groups ($p > 0.05$). However, there were significant differences in sex, smoking, diabetes, hypertension, and PCI history ($p < 0.05$). Results of laboratory tests showed that there were no significant dif-

ferences in the counts of RBC, lymphocytes, eosinophils, and basophils among 3 groups ($p > 0.05$). Compared with the control group and SAP group, white blood cells (WBCs), neutrophils, and monocytes in the ACS group were significantly increased ($p < 0.01$), as well as the value of PT, APTT, and DD dimers ($p < 0.05$). There were significant differences in HDL-C between all the groups ($p < 0.001$), and the highest level was in the control group. Cr, BUN, alanine aminotransferase (ALT), aspartate aminotransferase, total protein, albumin, direct bilirubin, and uric acid were all significantly different in the ACS group compared with the other 2 groups ($p < 0.05$). Compared with the control group, SAP and ACS groups had higher glucose ($p < 0.01$). Echocardiography showed there were significant differences in LVEF%, LVPW, and LA. The ACS group showed the lowest LVEF% ($p < 0.05$), the highest LVPW ($p < 0.001$), and the highest LA ($p < 0.05$).

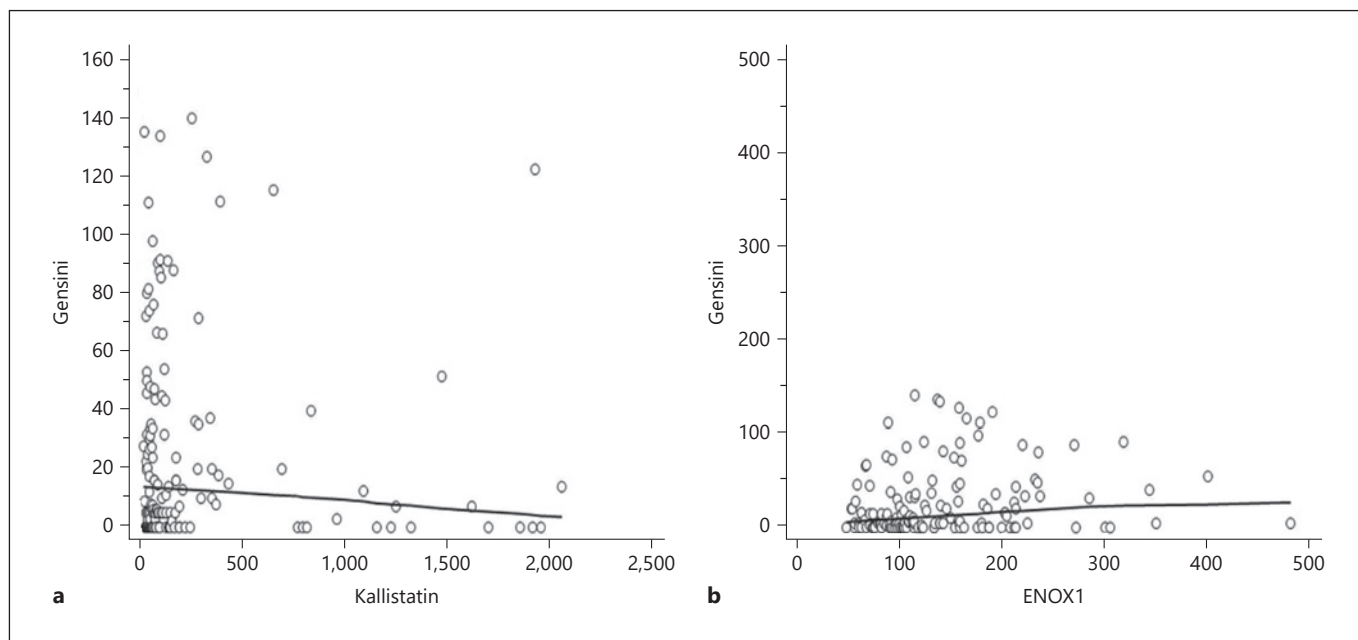


Fig. 2. Scatter (dot) plot with Pearson correlation analysis between kallistatin and Gensini score (a), as well as ENOX1 and Gensini score (b).

Table 3. Correlation analysis of circulating kallistatin and ENOX1 levels with cardiac risk factors

	Kallistatin		ENOX1	
	R	p value	R	p value
Gensini score	-0.193	0.017	0.178	0.028
WBC	-0.262	0.001	0.213	0.009
TG	-0.224	0.008	0.005	0.957
Age	0.360	0.000	0.049	0.549
HDL	0.303	0.000	-0.126	0.143
INR	-0.032	0.697	0.163	0.048

WBC, white blood cell; TG, triglyceride; INR, international normalized ratio.

Comparison of Serum Levels of Kallistatin and ENOX1 in CHD and Control Group

Compared with the control group, kallistatin level was significantly decreased in the SAP group and ACS group ($3,094.69 \pm 1,435.65$ vs. $2,564.15 \pm 955.32$ vs. $2,411.85 \pm 1,427.91$), and ENOX1 expression was significantly increased in ACS group (129.23 ± 79.43 vs. 119.07 ± 53.98 vs. 166.53 ± 74.49) ($p < 0.05$) (Table 2; Fig. 1). There were significant differences in Gensini scores among all groups ($p < 0.001$) with the highest in the ACS group.

Pearson Correlation Analysis of Kallistatin and ENOX1

Pearson correlation analysis (Table 3) showed that kallistatin expression was negatively correlated with the Gensini score ($r = -0.193$, $p = 0.017$), WBC count ($r = -0.262$, $p = 0.001$), and TG levels ($r = -0.224$, $p = 0.008$) and positively correlated with age ($r = 0.360$, $p < 0.001$) and HDL-C ($r = 0.303$, $p < 0.001$). ENOX1 expression was positively correlated with WBC count ($r = 0.213$, $p = 0.009$), INR ($r = 0.163$, $p = 0.048$), and Gensini scores ($r = 0.178$, $p = 0.028$). Scatter (dot) plot with Pearson correlation analysis between Kallistatin and Gensini score, as well as ENOX1 and Gensini score, were shown in Figure 2.

Multiple Linear Regression of Kallistatin and Gensini Score

Multiple linear regression showed that age, HDL-C, lipoa, and WBCs were the multiple factors influencing kallistatin level (Table 4). The fitting equation: [Kallistatin] = $41.364 \times \text{age} + 1,489.609 \times \text{HDL-C} - 0.832 \times \text{lipoa} - 70.589 \times \text{WBC} - 775.579$ ($R^2 = 0.308$, $F = 12.444$, $p < 0.001$). Cr, ALT, glucose, and kallistatin are independently related to Gensini scores (Table 5); [Gensini] = $0.434 \times \text{Cr} + 0.456 \times \text{ALT} - 0.005 \times \text{Kallistatin} + 1.798 \times \text{glucose} - 22.496$ ($R^2 = 0.46$, $F = 7.061$, $p = 0.009$).

Table 4. Multivariate linear regression analysis of kallistatin and related factors

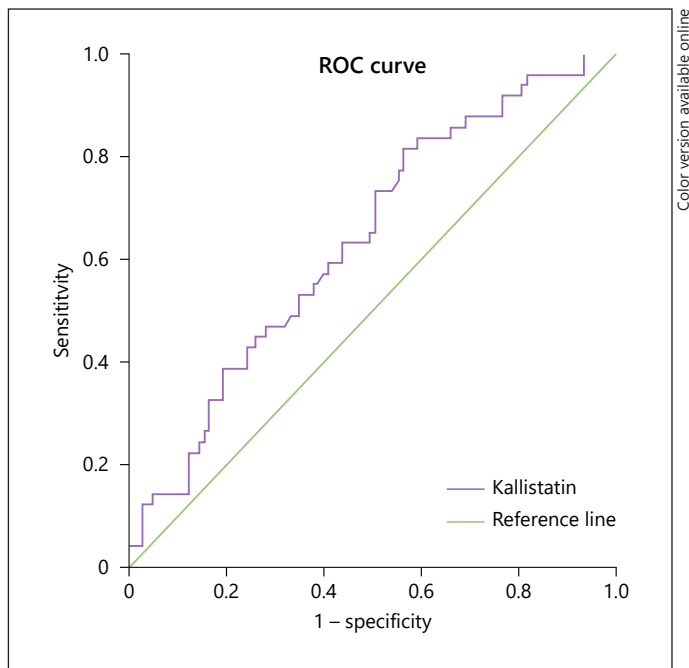
Factor	B	SD	Beta	t	95% CI	p value
Age	41.364	8.450	0.387	4.895	24.621–58.107	0.000
HDL	1,489.609	392.582	0.304	3.794	711.759–2,267.459	0.000
Lipoa	–0.832	0.318	–0.208	–2.617	–1.462 ~ –0.202	0.010
WBC	–70.589	35.173	–0.161	–2.007	–140.281 ~ –0.898	0.047
Constant	–775.579	802.302		–0.967	–2,365.238–814.079	0.336

Lipoa, lipoprotein a; WBC, white blood cell.

Table 5. Multivariate linear regression analysis of the Gensini score and related factors

Factor	B	SD	Beta	t	95% CI	p value
Cr	0.434	0.092	0.346	4.734	0.253–0.616	0.000
ALT	0.456	0.109	0.307	4.192	0.241–0.671	0.000
Kallistatin	–0.005	0.002	–0.195	–2.653	–0.009 ~ –0.001	0.009
GLU	1.798	0.766	0.170	2.346	0.282–3.313	0.020
Constant	–22.496	10.866		–2.070	–43.985 ~ –1.006	0.040

ALT, alanine aminotransferase.

**Fig. 3.** The ROC curve of kallistatin analyzed with 50 control subjects and 103 CHD patients. ROC, receiver operating characteristic; CHD, coronary heart disease.

ROC Curve of Kallistatin

The ROC analysis was used to examine the role of kallistatin as a biomarker for CHD. The results showed that the area under the curve of kallistatin was 0.636, with a sensitivity of 0.735 and a specificity of 0.495 (Fig. 3).

Discussion

CHD has become the most common disease and the leading cause of death around the world [15]. It is well known that atherosclerosis is the main pathological basis of CHD. Inflammation plays a primary role in the initial formation of atherosclerotic plaques and in the subsequent process of development into vulnerable plaques [16, 17]. Once the vulnerable plaque in the coronary artery ruptures, thrombi form in the arteries leading to ischemic necrosis of the myocardium, resulting in a spectrum of clinical presentations referred to as ACS [18]. Oxidative stress, a phenomenon induced by the excessive accumulation of reactive oxygen species including superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide, hydroxyl radicals ($\cdot OH$), and singlet oxygen (1O_2) in cells, usually damages important cellular elements such as proteins and nucleic acids [19]. Oxidative stress can stimulate the inflammatory response by increasing the levels of ex-

pression of proinflammatory cytokines [20]. Oxidative stress has been shown to be a major pathogenic factor in the progression of atherosclerosis [21]. Thus, research on oxidative stress and inflammation is of great significance in the diagnosis and treatment of atherosclerosis.

Kallistatin, an endogenous protein, could regulate a variety of cellular biological behaviors with its 2 special structural components: an active site and a heparin-binding domain. Kallistatin is widely expressed in multiple tissues and organs including heart and arterial vessels [22]. Recently, several researches revealed kallistatin to possess anti-inflammatory and anti-oxidative stress properties, thus inhibiting atherosclerotic plaque formation [23, 24]. ENOX1, an NADH oxidase, is localized on the surface of the plasma membrane and catalyzes the oxidation of both NADH or hydroquinone, as well as protein disulfide-thiol interchange [25]. Lee et al. [26] found that the plasma membrane electron transport (containing ENOX1) pathway is activated by oxidized-1-palmitoyl-2-arachidonoyl-sn-glycerol-3-phosphocholine (ox-PAPC) and then induces oxidative stress and upregulates the expression of heme oxygenase-1 (HO-1) in human aortic endothelial cell; it is suggested that ENOX1 may be a regulator of oxidative stress in endothelial cells and a potential pathogenic factor in atherosclerosis.

In our study, expression of kallistatin was lower in the SAP group and the ACS group compared to the control group, which indicated a potential anti-atherosclerotic role of kallistatin. ENOX1 expression was significantly higher in the ACS group compared with control group and SAP group. Considering the association between oxidative stress and plaque rupture and subsequent complications of myocardial ischemic infarction [27, 28], we speculate that the elevation of ENOX1 in the ACS group is mainly due to increased oxidative stress caused by acute coronary events and may act as an independent risk factor for MI.

We also explored the correlations between kallistatin, ENOX1 and CHD-related factors that were assessed in our study, including leukocyte, RBC, platelet, CK-MB, Myo, INR, PT, APTT, D-dimer, TG, total cholesterol, low-density lipoprotein cholesterol, HDL-C, apolipoprotein A1, apolipoprotein B, and blood glucose. We found that the expression of kallistatin was negatively correlated to WBC count and TG levels and is positively correlated to HDL-C, which further clarified that kallistatin may have a cardiovascular protective effect by regulating the inflammatory response and blood lipid level.

Next, we revealed a negative correlation between kallistatin and CHD Gensini scores, while ENOX1 was positively correlated with Gensini scores. At present, there is limited information about the mechanism of action of kallistatin and ENOX1 in atherosclerosis. First, kallistatin may increase the expression of endothelial protective factors such as nitrous oxide synthase, sirtuin1, interleukin-10, superoxide dismutase 2 and thus reduce the size of plaques in the carotid artery [23]. In addition, kallistatin helps to reduce the accumulation of macrophages, the degree of oxidative stress and inflammatory response in the vascular wall [23, 24]. In human aortic endothelial cells, ENOX1 could activate oxidative stress and has potential atherogenic effects [26]. In vivo experiments showed that anti-kallistatin promotes NADH oxidase activity and increases superoxide content in the heart [29]. Therefore, kallistatin may reduce oxidative stress partly by suppressing ENOX1, thus inhibiting the progression of atherosclerosis. Besides, kallistatin was positively correlated with age which is an independent risk factor for atherosclerosis [30]. Therefore, we speculate that the positive correlation between kallistatin and age may result from the compensatory mechanisms in CHD. Altogether, kallistatin and ENOX1 may interact with each other and influence the development of CHD.

Enhanced inflammatory response and oxidative stress are involved in CHD, especially in acute myocardial infarction [31, 32]. We have measured serum levels of kallistatin and ENOX1 in patients with different types of CHD. Dysregulated expressions of kallistatin and ENOX1 in CHD patients suggest that both of them may be related to atherosclerosis and may have potential value in predicting the severity of CHD. Although correlation analysis showed a negative correlation between kallistatin and Gensini score and a positive correlation between ENOX1 and Gensini score, multiple linear regression analysis further showed that kallistatin has significant predictive value for Gensini score, while ENOX1 does not. ROC analysis suggests that kallistatin may have diagnostic value in CHD.

Conclusion

Kallistatin was significantly decreased in patients with CHD (SAP and ACS), while ENOX1 was significantly increased in ACS. Kallistatin and ENOX1 are associated with the severity of CHD and kallistatin may serve as a novel biomarker for the diagnosis of CHD.

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Statement of Ethics

The study was approved by the Ethics Committee of Southeast University affiliated to Zhongda Hospital, and all participants provided informed written consent before they were included in the study.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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