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Original Article

Serum TIMP-2, NGAL and angiopoietin-2 as biomarkers of coronary artery stenosis

Yeoeun Han*, Hyung Joon Joo*, Ha-Rim Seo, Seung-Cheol Choi, Jae Hyoung Park, Cheol Woong Yu, Soon Jun Hong, Do-Sun Lim

*Department of Cardiology, Cardiovascular Center, Korea University Anam Hospital, Seoul, Korea. *Equal contributors.*

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Abstract: Background and Aim: Coronary artery atherosclerosis develops through the interplay of lipid metabolism, vascular endothelial activation and immune cell activation. In this study, newly discovered biomarkers for vascular inflammation and activation were tested for diagnostic strength in angiographically significant coronary artery stenosis. Methods: Serum levels of NGAL, TIMP2, IL-8, GRO alpha, angiopoietin-2, bFGF and hsCRP were measured in 70 patients undergoing coronary angiography. Severity of disease was evaluated from angiographic findings based on the modified Gensini score. Results: Serum TIMP-2, NGAL and angiopoietin-2 levels were significantly elevated in patients with coronary artery stenosis ($P = 0.002$, $P = 0.01$ and $P = 0.01$, respectively) and correlated significantly with the number of stenotic coronary arteries. Receiver operating characteristic analysis of TIMP-2, NGAL and angiopoietin-2 showed significantly increased areas under the curve (AUC = 0.795, 0.728, and 0.722, respectively). Multivariate analysis revealed that the elevated serum angiopoietin-2, old age and current smoking significantly and independently predicted the presence of coronary artery stenosis ($P = 0.022$, $P = 0.006$ and $P = 0.004$, respectively). Conclusion: Serum TIMP-2, NGAL and angiopoietin-2 levels may be useful in predicting the presence and severity of coronary artery stenosis.

Keywords: TIMP-2, NGAL, angiopoietin-2, coronary artery stenosis

Introduction

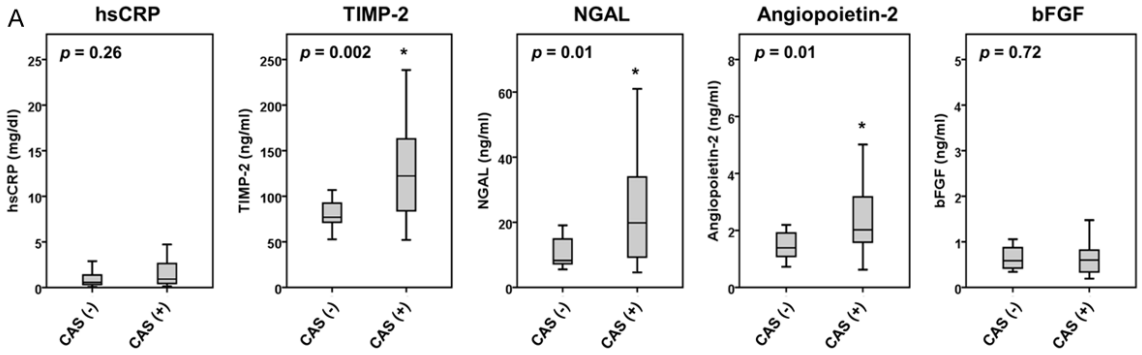
Coronary angiography may be regarded as the gold standard for diagnosis of coronary artery stenosis (CAS). Although an invasive endovascular technique, angiography only rarely results in serious complications such as vascular dissection or thrombosis [1, 2]. Among the non-invasive methods for assessment of CAS, stress tests, including the treadmill test, stress echocardiography, and myocardial perfusion scanning provide the mainstay. Overall accuracy of the CAS diagnosis by using those non-invasive methods remains unsatisfactory, however, as more than 30% of patients without suspected cardiac disease may be found by coronary angiography to have significant coronary artery stenosis [3]. The recent introduction of computed tomographic angiography (CTA), a non-invasive alternative to coronary angiography [4] may potentially extend the accuracy of CAS diagnosis. The overall sensitivity of CTA for coronary artery stenosis is reportedly as high as 85% to 95% and specificity, 90% to 97% [5, 6]. However, increases in heart rate and cardiac arrhythmia, use of a nephrotoxic contrast dye, and extensive radiation exposure related to CTA urge caution in its clinical application [7]. Technology with less hazardous, invasive and complex is needed to provide an accurate and acceptable population screening method for CAS.

As a multi-factorial time-dependent process, coronary atherosclerosis generates physiological artifacts, or biomarkers, well in advance of clinical disease [8, 9], and measures of these biomarkers in blood may indicate the status and predict the outcome of CAS. However, use of biomarkers in screening for coronary artery disease awaits thorough evaluation of their biological and clinical significance.

Table 1. Baseline patient characteristics

Variables	Total (n = 70)	Normal (n = 13)	1-vessel disease (n = 22)	2-vessel disease (n = 17)	3-vessel disease (n = 18)	*P*-value
Men/Women	57/13	11/2	19/3	12/5	15/3	0.65
Age (years)	58.5	52.3±10.0	57.0±9.4	56.8±9.8	60.4±9.8	0.07
Body mass index (kg/m^2)	24.0 (3.9)	24.0±3.2	23.4±2.9	24.9±4.1	25.5±3.1	0.82
Hypertension	39 (55.7%)	5 (38.5%)	11 (50.0%)	11 (64.7%)	12 (66.7%)	0.83
Diabetes mellitus	14 (20%)	1 (7.7%)	4 (18.2%)	4 (23.5%)	5 (27.8%)	0.16
Dyslipidemia	13 (18.8%)	5 (38.5%)	3 (13.6%)	3 (17.6%)	2 (11.8%)	0.14
Smoking status						0.74
Current smoker	30 (42.9%)	4 (30.8%)	11 (50.0%)	8 (47.1%)	7 (38.9%)	
Past smoker	16 (22.9%)	2 (15.4%)	6 (27.3%)	4 (23.5%)	4 (22.2%)	
Never smoker	24 (34.3%)	7 (53.8%)	5 (22.7%)	5 (29.4%)	7 (38.9%)	
LDL cholesterol (mg/dl)	91.2±30.1	102.4±31.4	85.9±32.8	86.4±28.3	89.0±31.7	0.61
Triglycerides (mg/dl)	125.0 (88)	186.0 (146)	126.9±68.1	116.0±43.5	119.0±40.9	0.61
Creatinine (mg/dl)	1.0 (0.3)	1.1±0.2	0.9±0.2	1.0±0.2	1.0±0.2	0.62
eGFR (ml/min/1.73 m^2)	77.5±14.6	68.7±9.1	81.9±19.2	71.6±10.6	76.6±11.9	0.28

Data are presented as the mean ± SD for continuous, normally distributed variables; median (interquartile range) for continuous, non-normally distributed variables; and number (percent) for categorical variables. eGFR, Estimated glomerular filtration rate.



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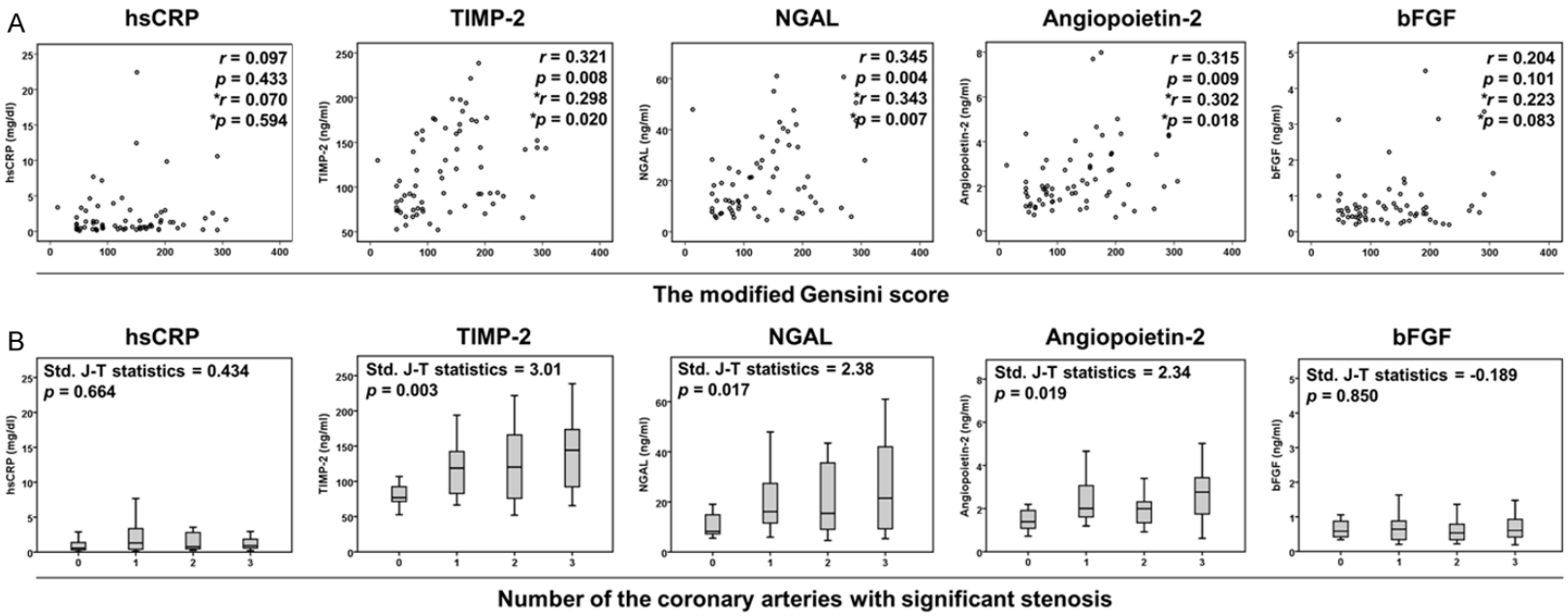
	hsCRP (mg/L)	TIMP-2 (ng/dL)	NGAL (ng/dL)	Angiopoietin-2 (ng/dL)	bFGF (ng/dL)
CAS (-)	1.2±1.3	80.6±17.0	11.7±6.7	1.6±0.9	0.6(0.5)
CAS (+)	0.9(2.2)	126.1(78.0)	21.4(27)	2.5±1.5	0.6(0.7)
***p*-value**	0.26	0.002	0.01	0.01	0.72

Figure 1. Serum TIMP-2, NGAL and angiopoietin-2 levels were significantly higher in patients with angiographically confirmed coronary artery stenosis. A. Graphs show mean values of serum hsCRP, TIMP2, NGAL, angiopoietin-2 and bFGF. B. Table shows data from figure above, presented as mean values ± SD for continuous, normally distributed variables and as median (interquartile range) for continuous or non-normally distributed variables. *$P < 0.05$; CAS, coronary artery stenosis confirmed by coronary angiography.

Vascular inflammation and angiogenesis appear to link the genetic and ecological components of CAD to the development and progression of vascular lesions. Biomarkers associated with vascular inflammation and angiogenesis, and coronary atherosclerosis include neutrophil gelatinase-associated lipocalin (NGAL), reported to be highly expressed in human arterial plaques [10]. Tissue inhibitor of metalloproteinase-2 (TIMP-2) may be associated with plaque stability [11]. Angiopietin-2 and basic fibroblast growth factor (bFGF) influence the stability of atherosclerotic plaque through endothelial cell and vascular smooth muscle



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Variables	Total (n=70)	Normal (n=13)	1-vessel disease (n=22)	2-vessel disease (n=17)	3-vessel disease (n=18)
hsCRP	0.9((1.8)	0.7(1.4)	1.4(2.9)	0.6(2.1)	0.9±0.7
New biomarkers					
NGAL (ng/mL)	14.9(19.9)	8.7(11)	18.5(16)	24.9±14.2	33.2±17.4
Angiopoietin-2 (ng/mL)	2.0(1.6)	1.8±1.0	2.4±1.1	2.0(2.5)	2.7±1.2
TIMP-2 (ng/mL)	101.1(76.9)	79.6±17.2	121.2±36.9	141.2±55.9	151.9±47.9
bFGF (ng/ml)	0.6(0.6)	0.9±0.9	0.7±0.4	2.0(2.5)	2.7(2.2)
The modified Gensini score	126.5(111)	56.5±11.7	91.0(76)	146.7±52.9	207.1±56.3

Figure 2. Serum TIMP-2, NGAL, and angiopoietin-2 levels were significantly correlated with angiographically assessed severity and number of vessels involved in coronary artery disease. (A and B) Scatter plots of serum hsCRP, TIMP-2, NGAL, angiopoietin-2 and bFGF against the modified Gensini score (A) and number of coronary arteries with significant stenosis (B). (C) Table shows data from figures above, stratified by number of coronary arteries with angiographically significant stenosis. Data are presented as the mean ± SD for continuous, normally distributed variables and as median (interquartile range) for continuous or non-normally distributed variables. *, value adjusted for age and sex using partial correlation analysis; Std. J-T statistics, standard J-T statistics.

Table 2. Correlations between serum TIMP-2, NGAL, angiopoietin-2 and bFGF concentrations

	Correlation coefficient	*P*-value
TIMP-2 vs. NGAL	0.620	< 0.01
TIMP-2 vs. Angiopoietin-2	0.578	< 0.01
Angiopoietin-2 vs. NGAL	0.463	< 0.01
Angiopoietin-2 vs. bFGF	0.237	0.056
TIMP-2 vs. bFGF	0.231	0.062
NGAL vs. bFGF	0.173	0.168

cell activation [12, 13]. Growth-regulated protein alpha (GROα) and interleukin-8 (IL8) are associated with immune cell adhesion to atherosclerotic plaque [14].

The purpose of this study was to test a panel of biomarkers related to immune cell recruitment and plaque inflammation (NGAL, TIMP2, IL-8, and GROα), as well as vascular activation (Angiopoietin-2 and bFGF) for diagnostic significance in coronary artery disease.

Materials and methods

Study subjects

Patients with clinical suspicion of coronary artery disease who were admitted to our clinic for the elective coronary angiography were prospectively selected for this study. The exclusion criteria were acute coronary syndrome, acute infection, autoimmune disease and inflammatory disease, significant hepatic or renal diseases and anti-inflammatory drug use. In 70 patients, coronary artery stenosis was confirmed by coronary angiography. These patients were enrolled for study through the department of cardiology at Korea University Anam Hospital (Seoul, South Korea) beginning in July 2012 and ending in March 2013. The ethics committees of Korea University Anam Hospital approved this study. All patients gave their informed consent to use part of their blood for scientific purposes.

Coronary angiography interpretation

Significant coronary artery stenosis was defined as luminal stenosis of at least 50% in more than one major coronary artery. Measurement of coronary artery disease burden was based on the modified Gensini score [15, 16].

Measurements of serum NGAL, angiopoietin-2, TIMP2, bFGF, IL8, GRO alpha

Blood samples were obtained in EDTA tubes just before coronary angiography. Each serum sample was divided into three aliquots and stored at -80°C until analysis. Repeated freeze-thawing was avoided. Serum NGAL, angiopoietin-2, TIMP2, bFGF, IL8 and GROα were measured by enzyme-linked immunosorbent assays (ELISA) using commercially available kits (Raybiotech, Inc., GA, USA) according to the manufacturer's instructions. All samples were assayed in duplicate by a researcher who was blinded to sample identities.

Other laboratory parameters including high-sensitive C-reactive protein (hsCRP), creatinine, and lipids were evaluated at the chemistry laboratory of Korea University Anam Hospital.

Statistics

Median values with interquartile range are reported for continuous variables, and counts with percent for categorical variables. The Kolmogorov-Smirnov test was used to test continuous variables for normality. Comparisons between two groups were performed using Student's t-tests or a Mann-Whitney test for continuous variables, and chi^2 tests or Fisher's exact test for categorical variables. The Jonckheere-Terpstra test was used to compare data from more than two groups. Correlations between variables were tested using Pearson's method. Factors predicting significant coronary artery stenosis were tested using multiple stepwise logistic regression analysis. Variables tested for predictive power included age, sex, hypertension, diabetes, dyslipidemia, smoking, body mass index, estimated glomerular filtration rate and serum creatinine, NGAL, TIMP-2, angiopoietin-2, bFGF and hs-CRP concentrations. Receiver operating characteristic (ROC) curves were constructed for the diagnosis of significant coronary artery stenosis. Two academic authors analyzed the database independently and reconciled any differences. All tests were two-sided and a *P*-value less than 0.05 was considered to indicate significance. All calculations were performed using the Statistical Package for the Social Sciences (SPSS) software (Version 18.0, SPSS Inc., Chicago, IL, USA).

Table 3. Correlations between proposed biomarkers and clinical variables

		TIMP-2	NGAL	Angiopoietin-2	bFGF	hsCRP
Age	Correlation coefficient	0.060	-0.040	-0.108	-0.311	0.091
	P-value	0.624	0.747	0.386	0.011	0.462
Sex	Correlation coefficient	0.284	0.163	0.237	0.152	-0.135
	P-value	0.019	0.188	0.54	0.222	0.274
BMI	Correlation coefficient	0.238	0.292	0.192	0.108	0.056
	P-value	0.050	0.017	0.120	0.387	0.650
Hypertension	Correlation coefficient	0.269	0.134	0.91	0.006	0.169
	P-value	0.027	0.278	0.465	0.960	0.168

Table 4. Factors predicting a diagnosis of angiographically significant coronary artery stenosis

	OR (95% CI)	*P*-value
Angiopoietin-2	5.408 (1.279-22.865)	0.022
Age	1.261 (1.068-1.489)	0.006
Current smoking	66.311 (3.793-1159.318)	0.004
Body mass index	1.579 (0.993-2.511)	0.54

OR, Odds ratio; 95% CI, 95% confidence interval.

Results

Baseline demographic characteristics, stratified by number of coronary arteries with angiographically significant stenosis, are presented in **Table 1**. The mean age of participants was 58.5±10.2 years; 81% were male, 55.7% had hypertension, 20% had type 2 diabetes, and 18.8% had hypercholesterolemia.

We measured the serum hsCRP, TIMP-2, NGAL, angiopoietin-2, bFGF, IL-8 and GROα. The IL-8 and GROα were not analyzed further because IL-8 levels in 20 samples and GROα levels in 59 samples were below the detection limits. TIMP-2, NGAL and angiopoietin-2 levels were significantly elevated in patients with coronary artery stenosis (P = 0.002, P = 0.01 AND P = 0.01, respectively; **Figure 1**).

The serum TIMP-2, NGAL and angiopoietin-2 levels were also significantly correlated with the number of stenotic coronary arteries and the angiographical severity based on the modified Gensini score (**Figure 2**). When adjusted for age and sex, these markers still correlated significantly with severity of stenosis by angiography. The strongest associations were observed between NGAL and the modified Gensini score (r = 0.345, P = 0.004 (crude); *r = 0.343, P = 0.007 (adjusted)).

The serum TIMP-2, NGAL, angiopoietin-2, and bFGF levels correlated significantly with each other (**Table 2**). The correlation coefficient for TIMP-2 and NGAL was highest (r = 0.620, P < 0.01).

Correlations between biomarkers and clinical variables are shown in **Table 3**. The TIMP-2 level was statistically correlated with sex (r = 0.284, P = 0.019) and hypertension (r = 0.269, P = 0.027). NGAL was correlated with body mass index (r = 0.292, P = 0.017), and bFGF with age (r = -0.311, P = 0.011). Other clinical variables did not correlate significantly with TIMP-2, NGAL, angiopoietin-2, bFGF or hsCRP.

In multiple step-wise logistic regression analyses, angiopoietin-2, age and current smoking were predictive for angiographically significant coronary artery stenosis (P = 0.22, P = 0.006 AND P = 0.004, respectively; **Table 4**).

Based on the receiver operating characteristic curves, we analyzed the accuracy of these putative biomarkers in predicting angiographically significant coronary artery stenosis. ROC curves of TIMP-2, NGAL, angiopoietin-2, bFGF, and hsCRP are shown in **Figure 3A**. The areas under the curve (AUC) were significantly greater for TIMP-2, NGAL and angiopoietin-2 (**Figure 3B**). Using a cut-off value of 77.87 ng/ml for TIMP-2, a sensitivity of 80.0% and specificity 53.8% were obtained while a cut-off value of 91.46 ng/ml produced 67.3% sensitivity and 69.2% specificity. A cut-off value of 9.15 ng/ml for NGAL gave 77.4% sensitivity and 61.5% specificity, while a cut-off at 15.15 ng/ml showed 56.6% sensitivity and 76.9% specificity. A cut-off value of 1.57 ng/ml angiopoietin-2 showed 75.5% sensitivity and 61.5% specificity, and a cut-off value of 1.91 ng/ml yielded 56.6% sensitivity and 76.6% specificity.

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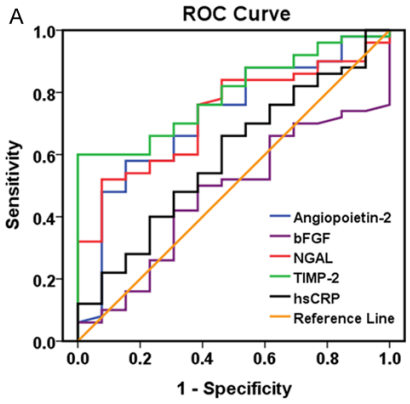

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	AUC	*p*-value
TIMP-2	0.795	0.010
NGAL	0.728	0.012
Angiopoietin-2	0.722	0.014
hsCRP	0.594	0.300
bFGF	0.469	0.734

Figure 3. ROC analysis of biomarker performance in the diagnosis of the angiographically significant coronary artery stenosis. A. ROC curves for serum hsCRP, TIMP2, NGAL, angiopoietin-2 and bFGF. B. Table shows biomarker sensitivities and specificities derived from ROC analysis.

Discussion

The principal aim of this study was to evaluate putative biomarkers of inflammation, angiogenesis, and plaque remodeling for the power to predict coronary artery stenosis as confirmed by coronary angiography. The main findings of the study were as follows: 1) serum levels of TIMP-2, NGAL and angiopoietin-2 were significantly higher in patients with coronary artery stenosis; 2) serum levels of TIMP-2, NGAL and angiopoietin-2 were correlated with coronary artery disease severity; 3) TIMP-2, NGAL and angiopoietin-2 in patient serum showed moderate diagnostic accuracy in screening for coronary artery disease; 4) angiopoietin-2 was a significant and independent predictor of coronary artery stenosis.

The role of cholesterol metabolism in atherosclerotic coronary artery disease is complex. A high serum cholesterol level, or hypercholesterolemia, is associated with coronary atherosclerosis and its progression, and the low-density lipoprotein-cholesterol (LDL-C) fraction shows an especially strong correlation. Statins are shown to lower LDL-C levels, and to modify other components of arterial disease. Randomized controlled trials demonstrate that statin drugs lower LDL-C levels and reduce the risk of cardiovascular events in coronary artery disease [17].

High-sensitivity C-reactive protein (hsCRP), a nonspecific marker of inflammation, is one of the most extensively studied biomarkers for coronary artery disease, notably because elevation of serum hsCRP is associated with the coronary atherosclerosis and with risk for a cardiovascular event [18]. Evidence from the JUPITER trial supports combined testing for hsCRP and LDL-C to evaluate risk and guide preventive treatment for cardiovascular disease. Trial findings also support the anti-inflammatory action of statins within the cardiovascular system. Despite the benefits of statins, there is a considerable residual risk for cardiovascular events in coronary artery disease [19].

As risk indicators, LDL-C and hsCRP also show limited value in diagnosis [20-22]. In the present study, the serum hsCRP level was not associated with the presence of coronary artery stenosis or its severity. One possible explanation for the poor correlation of those conventional biomarkers in the diagnosis of coronary artery disease is that the wide use of statins for cardiovascular risk prevention in the enrolled patients may affect and lower their statistical correlation.

Research into the pathogenesis of coronary atherosclerosis reveals serum proteins that may potentially be used to diagnose and monitor coronary artery disease [23]. Through a literature review we identified TIMP2, NGAL, angiopoietin-2, bFGF, IL8 and GROα as serum proteins with plausible involvement in development and progression coronary atherosclerosis. Of these, we detected TIMP2, NGAL, angiopoietin-2 and bFGF in all of the patients in this study. In patients with angiographically confirmed coronary artery stenosis, serum levels of TIMP2, NGAL and angiopoietin-2 were significantly higher than in patients without angiographically confirmed coronary artery stenosis.

TIMP-2, an inhibitor of endogenous matrix metalloproteinases (MMPs), is associated with both vascular inflammation and platelet activation during atherosclerosis. In patients with stable coronary artery disease, serum levels of TIMP-2 and MMP-9 may increase [24]. In patients with early coronary atherosclerosis, on the other hand, plasma TIMP-2 levels may decrease [25].

NGAL, which is expressed in neutrophils and associated with degradation of MMP-9, influences remodeling of atherosclerotic plaque. Serum NGAL is reported to increase in coronary artery disease and to correlate with disease severity [26].

Angiopoietin-2 is selectively expressed in coronary endothelial cells and is related to integrity of the coronary artery endothelium, as shown in a porcine model system [27]. Plasma angiopoietin-2 may increase in patients with acute coronary syndrome [28]; however, recent study shows that serum angiopoietin-2 level may also increase significantly in patients with stable coronary heart disease [29].

In the present study we compared serum levels of several proposed biomarkers and hsCRP between patients with and without angiographically significant coronary arterial stenosis. While serum concentrations of TIMP-2, NGAL and angiopietin-2 were significantly higher in the patients with significant stenosis, serum hsCRP was not (**Figure 1**). Moreover, serum levels of TIMP-2, NGAL and angiopietin-2 correlated closely with the angiographically assessed severity of the stenosis (**Figure 2**).

In the present study, we tested various scoring methods to improve diagnostic accuracy results for TIMP-2, NGAL and angiopietin-2 but without success (data not shown). This may plausibly be attributed to the low specificity of the roles those biomarker proteins play in the development of atherosclerosis. Moreover, the close correlations among those markers (**Table 2**) suggested that those biomarkers act by the same or closely related pathogenic mechanisms. Based on a recent study, the plasma biomarkers monocyte chemoattractant protein-1, galectin-3 and N-terminal fragment of brain natriuretic peptide may independently and significantly predict cardiovascular events in patients with high-risk coronary artery disease [30]. Possibly the combination of one or more of those markers with markers investigated in the present study would improve the accuracy of our diagnostic panel. Meanwhile, identification of biomarkers that specifically represent the molecular and cellular mediators of atherosclerosis remains an important research target.

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Disclosure of conflict of interest

None.

Address correspondence to: Do-Sun Lim, Department of Cardiology, Cardiovascular Center, Korea University Anam Hospital, 126-1, 5ka, Anam-dong, Sungbuk-ku, Seoul 136-705, Korea. Tel: +82-2-920-5445; Fax: +82-2-927-1478; E-mail: dslmd@kumc.or.kr

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