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Substance P and Neuropeptide Y as Potential Biomarkers for Diagnosis of Acute Myocardial Infarction in Korean Patients

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Substance P and neuropeptide Y were discovered as early diagnostic biomarkers of acute myocardial infarction in Korean patients and confirmed using enzyme-linked immunosorbent assay (ELISA). We screened 12 peptides from the sera of Korean acute myocardial infarction (AMI) patients and detected 3 peptides (neuropeptide Y, substance P, and N-terminal pro-B-type natriuretic peptide) to be elevated from patients' sera by liquid chromatography mass/mass spectrometry. The elevated concentration of 3 peptides was confirmed by ELISA. The screening results revealed the substance P, neuropeptide Y, and pro-B-type natriuretic peptide (47-76) concentrations were higher in patients' sera than in healthy controls. The sensitivity and specificity of substance P for AMI diagnostic marker were 80% and 83%, respectively, and those of neuropeptide Y were 87% and 90%, respectively compared to healthy controls. These results suggest that substance P and neuropeptide Y could be used as early diagnostic biomarkers in patients with AMI.

Key Words : Substance P, Neuropeptide Y, Biomarker, Acute myocardial infarction, LC-MS

Introduction

Acute myocardial infarction (AMI) is associated with the highest mortality rate of patients among various cardiovascular diseases (CVD), which are the third leading cause of death in Korea.[1] Therefore, early and accurate diagnosis is very important for AMI patients. CVD includes coronary heart disease, cardiomyopathy, hypertensive heart disease, heart failure, cor-pulmonale, cardiac dysrhythmia, inflammatory heart disease, valvular heart disease, stroke, and cerebrovascular disease.[2-4] In particular, AMI is commonly known as a heart attack, which results from the interruption of blood supply to a part of the heart and causes the heart cells to die.[5] Currently, AMI is diagnosed on the basis of with a combination of 3 different characteristics, *i.e.*, severe chest pain, abnormal change on the electrocardiogram finding, and increased levels of cardiac serum biomarkers. However, chest pain can be checked but does not give a diagnostic result, and the electrocardiogram has been often non-diagnostic[6] and symptoms have been observed in less than 80% of positive AMI patients.[5] Thus, biomarkers are increasingly used in the clinical field for the diagnosis of AMI. Several serum biomarkers associated with AMI have been identified.[7,8] The gold standard for the diagnosis of AMI is cardiac troponin (cTn).[9] The CTn has been validated as a biomarker for the diagnosis of AMI[10] and also it is regarded as the most cardiac specific of available markers for myocardial damage.[11] The CTn assay was performed with a sandwich immunoassay using monoclonal capture antibodies. The lower limit of detection was 0.006 μg/L.[12] Monitoring myocardial infarction using American Heart Association criteria was required commercially available troponin tests.[13]

Therefore, cTn is a sensitive indicator of AMI (sensitivity: about 80%). However, it doesn't particularly specific (specificity: about 67%)[9] caused by a delayed increase of circulating levels.[14] Also, it doesn't have clear release kinetics and enough analytical reference.[5] Similar observations have been confirmed previously for creatine kinase MB (CK-MB).[15] CK-MB have increased the diagnostic value,[11] but it exists in small quantities in the serum and gives low specificity (about 55%).[16,17] Myoglobin is the first biomarker released a few hours after AMI, but it has a low specificity (43%) because of its rapid excretion into urine[4,18-20] and not cardiac-specific.[11] These are clearly limits the diagnostic value of cTn, CK-MB, myoglobin, and in the early phase of AMI for diagnosis.

Recently, some prognostic biomarkers of peptide such as N-terminal pro-B-type natriuretic peptide (NT-proBNP) were suggested for the diagnosis of AMI from the serum of patients.[21] Similar peptide markers, including atrial natriuretic peptide, B-type natriuretic peptide (BNP), and C-type natriuretic peptide have been studied extensively.[22] Currently, BNP, which is a peptide hormone released from cardiomyocytes upon a mechanical stretch, has been recommended for clinical use.[21,23] It is processed by furin and its inactive N-terminal (NT) fragment is NT-proBNP.[23] An NT-proBNP of 8.5 kDa is now successfully used as a marker for congestive heart failure.[24] NT-proBNP is released into the plasma predominantly from ventricular cardiomyocytes, particularly in patients with chronic cardiac diseases.[16,17] It is a more sensitive and specific biomarker of ventricular dys-

function than the active BNP.[23] Indeed, recent studies have shown that assessment of the NT-proBNP concentration is useful in identifying cardiac disease.[21,25] Unfortunately, the area under the receiver operating characteristic (ROC) curve was below 70% and sensitivity and specificity values were below 80%.[26,27] Therefore, the development of a more sensitive and specific diagnostic biomarker is necessary for the early diagnosis of AMI.

In addition, angina pectoris (AP) is commonly known as angina following chest pain caused by ischemia of the heart muscle, which is in general due to obstruction or spasm of the coronary arteries. Unstable angina (UA) is also defined as AP caused by disruption of an atherosclerotic plaque with partial thrombosis and possible embolisation or vasospasm.[28] So far, AP and UA are difficult to be diagnosed.

In this work, we have screened 2 novel biomarker candidates in the serum of patients with AMI and validated them by comparing clinical patients with healthy controls using commercially available enzyme-linked immunosorbent assay (ELISA) kits.

Experimental

Materials and Sample Preparation. We analyzed 29 healthy controls and 81 patient samples. The serum was collected with written informed consent of the patients at the Korea University Medical Center (Seoul, Korea). The serum samples were collected within 24 h after the symptom onset. The Institutional Review Board of Korea University Medical Center approved the sample collection and analysis. The serum was collected from patients with AMI (n = 30), UA (n = 21), and AP (n = 30) together with healthy controls (n = 29). The collected blood samples were centrifuged for 40 min at 10000 × *g*, 4 °C, and stored at −70 °C until testing after addition of 0.6 TIU/mL aprotinin. For cTn analysis, the laboratory standard protocol of a chemical luminal immunoassay was done in Korea University College of Medicine.

We used 3 kinds of commercially available ELISA kits. The substance P (SubP) ELISA kit was purchased from R&D Systems, Inc. (Minneapolis, USA). The NT-proBNP ELISA kit was purchased from Biomedica Slovakia spol s.r.o. (Bratislava, Slovakia). The neuropeptide Y (NPY) ELISA kit was purchased from Phoenix Pharmaceuticals Inc. (Burlingame, USA). Triple distilled water (18.3 MΩ·cm) was prepared using Millipore® Synergy (Molsheim, France). The peptide standards-neuropeptide Y, *N*-formyl-Met-Leu-Phe (NFMLP), and a fragment of NT-proBNP (BNP(47-76)) were purchased from Phoenix Pharmaceuticals (Burlingame, USA). SubP was purchased from Sigma-Aldrich (St. Louis, USA). Analytical grade organic solvents were purchased from J.T. Baker (Phillipsburg, USA or Center Valley, USA). Formic acid (FA) was purchased from Sigma-Aldrich (Rockford, USA).

Extraction of peptides from serum was accomplished by the solid phase extraction method. The extraction of peptides from serum was done using Oasis HLB cartridge (Wexford, Ireland) according to the manufacturer's protocol after 4-fold dilution of the samples with acidic buffer (10% FA in distilled water (v/v)). The extracted samples were dried by using a nitrogen evaporator and a speed vacuum centrifuge connected with a freeze dryer. The dried samples were reconstituted with 100 μL of 50% aqueous methanol containing 0.2% FA. Alpha 1-2 LD plus freeze dryer (Marin Christ, Osterode am Harz, Germany) and a Stuart sample concentrator (Bibby Scientific Limited, Staffordshire, UK) were used.

Table 1. Gradient conditions used for the separation of target peptides in serum samples

Time (min)	Flow rate (mL/min)	solution A[a] (%)	solution B[b] (%)
Initial	0.35	100	0
1.00	0.35	100	0
8.00	0.35	80	20
10.00	0.35	65	35
12.00	0.3	60	40
15.00	0.35	30	70
17.00	0.35	0	100
18.50	0.35	0	100
19.00	0.35	100	0
23.00	0.35	100	0

[a]90% distilled water + 10% acetonitrile solution including 0.2% formic acid. [b]10% distilled water + 90% acetonitrile solution including 0.2% formic acid

Liquid Chromatography Mass/mass Spectrometry (LC-MS/MS) Analysis. For the analysis of peptides, ACQUITY UPLC (Waters, Milford, USA) and Thermo LTQ Orbitrap XL (Waltham, USA) were used. Chromatography was performed with a BEH C18 column (Waters). The particle size, diameter, and length of the column were 1.7 μm, 2.1 mm, and 100 mm, respectively. Table 1 showed the used gradient conditions for the separation and detection of targeted peptides. The 90% aqueous acetonitrile solution (solution A) and 10% aqueous acetonitrile solution (solution B) containing 0.2% FA were used. For the screening of known peptides and their identification in serum, the fragment scans mode was used. We screened 12 target peptides related to aging using the LC-MS/MS method published in elsewhere with slight modification.[29]

ELISA-based Confirmation of Biomarker Candidates. The SubP and NPY immunoassay kits are based on the principle of the competitive binding assay and NT-proBNP immunoassay kit utilized a sandwich binding assay method for the determination of the peptides in human serum. To construct a calibration curve, standard stock solutions were prepared from each peptide standard solution and serially diluted. We used the microplates coated with goat anti-mouse polyclonal antibody, polyclonal IgG secondary antibody, and polyclonal sheep anti-NT-proBNP antibody for tests of SubP, NPY, and NT-proBNP ELISA immunoassay.

The quality control samples and calibration standards were analyzed in duplicate. The serum samples were analyzed in triplicate after 2-fold dilution. The immunoassay has been done according to the manufacturer's protocols.

Instrumentation and Statistical Analysis. The optical

density (O.D.) was measured using a microplate reader (Bio-Rad, Hercules, USA). The absorbance (O.D.) was read at 450 nm. Diagnostic criteria, sensitivity, and specificity were statistically analyzed using MedCalc software (version 12.3, Mariakerke, Belgium). The correlation coefficients (R^2) and group differences were calculated by using the Student t-test. The ROCs were used for determining sensitivity and specificity. The statistical significance was considered when the P value was less than 0.05. The average and standard deviation of serum concentrations were calculated using the Excel 2007 Program (Microsoft, Washington, USA).

Results and Discussion

Screening of Peptidomic Biomarker Candidates for AMI Patients by LC-MS/MS.

For the development of peptidomic biomarker candidates, we screened 12 peptides which have been reported to be elevated in the elderly populations.[30,31] Among 12 targeted peptides, 4 peptides (NPY, NFMLP, SubP, and BNP(47-76)) were detected in the first screening of AMI patient samples. The qualitative analysis of peptide standards and patient samples showed same retention times and mass fragmentation patterns. The extracted total ion chromatograms and MS/MS fragmentation spectra of standard peptides and AMI serum samples are shown in Figure 1. The semi-quantitative analysis of NPY showed higher concentrations in AMI patient samples than that of controls, and NFMLP was detected in all serum samples (AMI patient, negative control, and gastric cancer patient; Figure 1(b-1)) with similar concentration. Trace

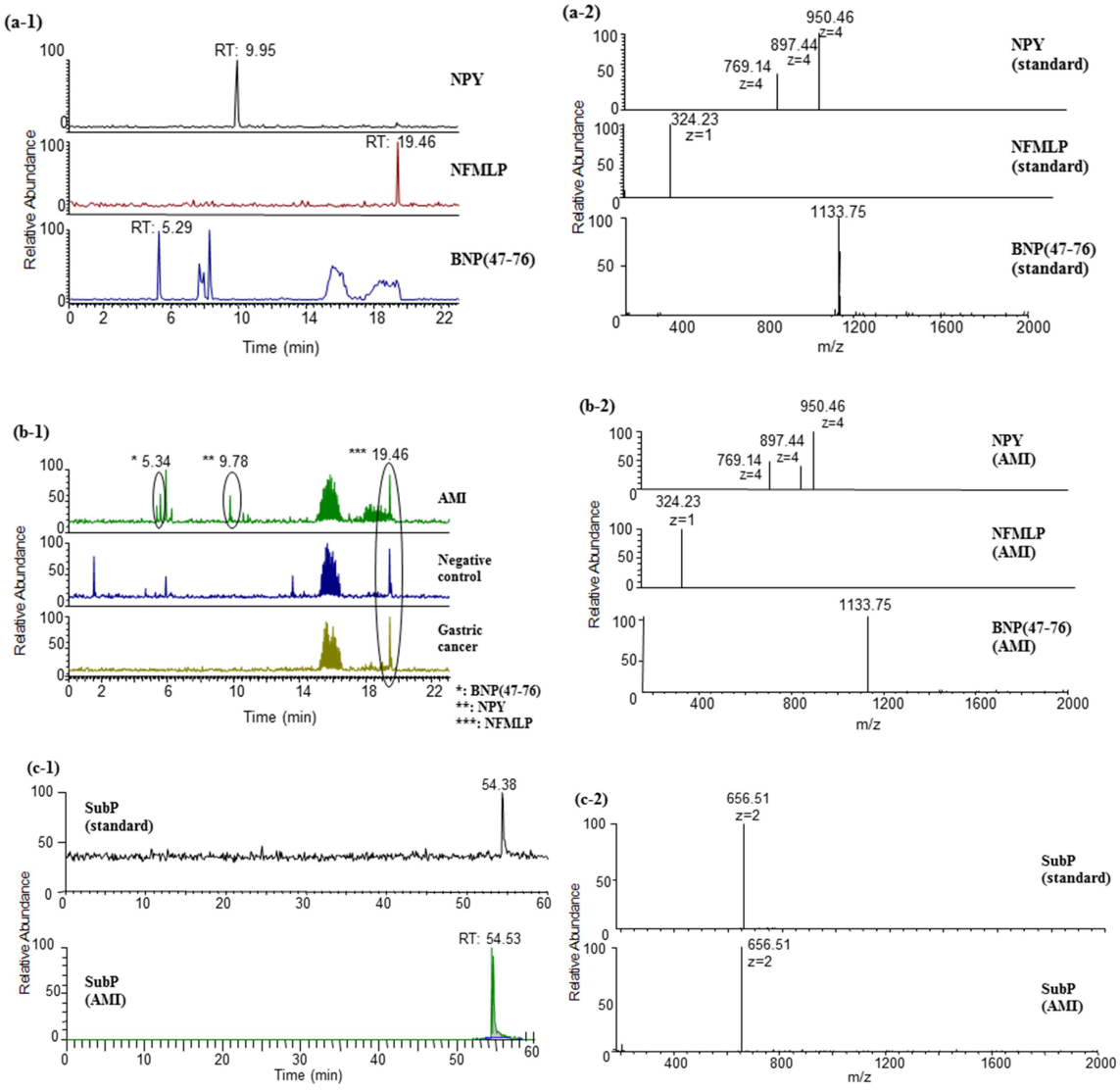

Figure 1. Screening results of peptidomic biomarker candidates for patients with acute myocardial infarction by liquid chromatography-tandem mass spectrometry; (a-1) Extracted ion chromatograms of NPY (retention time: 9.95 min), *N*-formyl-Met-Leu-Phe (retention time: 19.46 min), and pro-B-type natriuretic peptide (47-76) (retention time: 5.29 min) standards, and (a-2) MS/MS product ion mass spectra of standards. (b-1) Total ion chromatograms of 12 target peptides from serum samples of the acute myocardial infarction patient, negative control, and gastric cancer patients (*: BNP (47-76), **: NPY, ***: *N*-formyl-Met-Leu-Phe), and (b-2) MS/MS product ion mass spectra of serum samples with AMI patients (c-1) Extracted ion chromatogram of SubP (retention time: 54.38 min) standard, and serum samples with AMI patients (retention time: 54.53 min), (c-2) MS/MS product ion mass spectra of SubP standard, and serum samples with AMI patients.

amount of SubP and BNP(47-76) were detected in serum samples of the AMI patient and not in control samples (Figure 1(c-1) and (b-1)). The retention times of NPY, NFMLP, BNP(47-76), and SubP were 9.95, 19.46, 5.29, and 54.38 min, respectively (Figure 1(a-1) and (c-1)). The 5 ng/mL of reference standards was analyzed. In case of SubP, 4-fold concentrated samples could be detected its retained endogenous peptide (shown in Figure 1(c-1 and 2)). The characteristic fragment ions of NPY were 950.46 (z=4), 897.44 (z=4), and 769.14 (z=4). The major fragment ion was 324.23 (z=1) and 656.51 (z=2) for NFMLP (Figure 1(a-2 and b-2)) and SubP (Figure 1(c-2)), respectively. The fragmentation pattern of BNP(47-76) was different from sample to sample, but we could detect 1133.75 as major fragment ion from both of reference standard and the sample (Figure 1(a-2 and b-2)). SubP is known to be secreted from primary sensory afferent neurons after stimulation through protease-activated receptor 2.[32,33] The binding of SubP on neurokinin 1 receptors in neurons was reported to transmit pain signals through the central nervous system.[32] The role of SubP in cancer progress and immune response has been studied and the correlation between SubP levels and pain in chronic pancreatitis patients has been also reported.[34] Although the role of these molecules in other diseases was demonstrated previously, the possible contribution in AMI has not been studied.[35] The NPY is a 36-amino acid peptide neurotransmitter found in the brain and autonomic nervous system. It regulates energy usage, and is involved in learning, memory processing, and epilepsy.[36] It has been shown that NPY is expressed in the mammalian myocardium and that it co-localizes with norepinephrine in perivascular sympathetic neurons. NPY is released together with norepinephrine during conditions of high sympathetic activity and it contributes to the regulation of myocardial contractility through NPY receptor type 1 and type 2. The best known function of NPY is inhibition of neurotransmitter release, vasoconstriction and regulation of appetite.[37] The elevated levels of NPY receptor have been found in breast cancer, neuroendocrine tumors, kidney cancer, prostate cancer, and some types of sarcomas.[38] Until now, SubP and NPY have not studied as biomarkers for AMI, so we validated two biomarker candidates using human serum samples of the AMI patient by analysis of the ELISA method.

Validation of SubP and NPY as Biomarkers of AMI using ELISA. We analyzed serum samples of AMI, UA, and AP patients together with healthy controls using commercially available ELISA kits. The dynamic range of the used kit was 0-2500 pg/mL for SubP, 0-100 ng/mL for NPY, and 0-5424 pg/mL for NT-proBNP. The detection limit of quantifications (LOQ) was 31.5 pg/mL (SubP), 170 pg/mL (NPY), and 25.43 pg/mL (NT-proBNP). The accuracy of the assay method was within 8.4% intra-assay, within 15% inter-assay for SubP. The accuracy was within 10% intra-assay, within 15% inter-assay for NPY, and the accuracy was within 7% intra-assay, within 12% inter-assay for NT-proBNP. The recovery of the assay method was 82-117% (SubP), and 93-118% (NT-proBNP). The obtained calibration equations are shown in Table 2. The correlation coefficient was higher than 0.99 in all assays.

The information for patients is summarized in Table 3. Serum samples of 29 healthy controls and 81 patients were analyzed. The mean age and gender ratio were 44 ± 15 (male/female, 15/14), 56 ± 9 (24/6), 55 ± 11 (11/10), and 58 ± 10 (13/17), for the control, AMI, UA, and AP groups, respectively. The SubP concentration in AMI patients showed 2.7-fold higher than that of controls (191 ± 141 *vs.* 70 ± 131 pg/mL). The NPY concentration in AMI patients was found to be elevated in 2-fold compared to a healthy control group (1128 ± 781 *vs*. 435 ± 107 pg/mL). The NT-proBNP concentration in AMI patients was 1.9-fold higher than that of controls (459.86 ± 426 *vs*. 242 ± 72 pg/mL). The concentrations of SubP, NPY, and NT-proBNP in the serum of AMI patients were significantly higher than those in healthy controls (Figure 2, $P < 0.05$). Otherwise, the concentrations of UA or AP patients in the serum were not higher than those of healthy controls significantly for marker candidates.

Table 2. Calibration standard equations, correlation coefficients (R^2), and limit of quantifications (LOQ) measured by enzyme-linked immunosorbent assays

Parameters	SubP	NPY	NT-proBNP
Equation	Log(y) = –0.0003464x –0.02897	y = –0.9695Log(x) + 1.1770	y = 0.002939x + 0.1732
R^2	0.9977	0.9979	0.9934
LOQ (pg/mL)	31.5	170	25.43

Table 3. Characteristics of controls and subjects with acute myocardial infarction (AMI), unstable angina (UA), and angina pectoris (AP) (n=110)

Characteristics	Control	AMI	UA	AP
Age (years old)	44 ± 14	56 ± 9	55 ± 11	58 ± 10
Number of patients (male/female)	29 (15/14)	30 (24/6)	21 (11/10)	30 (13/17)
Conc. of SubP (pg/mL)	70 ± 131	191 ± 141	103 ± 111	31 ± 80
Conc. of NPY (pg/mL)	435 ± 107	1128±781	599 ± 415	525 ± 557
Conc. of NT-proBNP (pg/mL)	242 ± 71	486 ± 426	298 ± 188	269 ± 88
Hypertensive patients (number)	3	6	6	8

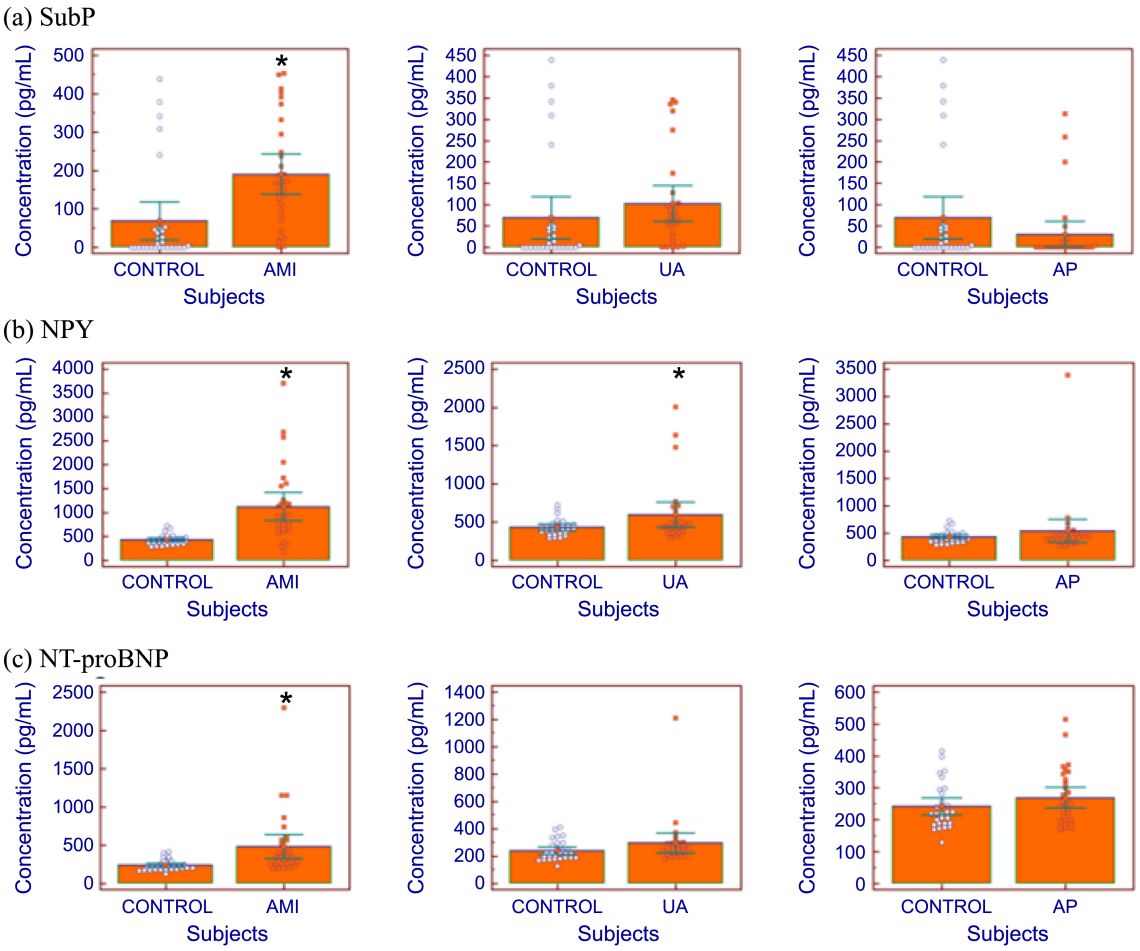

Figure 2. Comparison of marker candidate concentrations of patients with acute myocardial infarction, unstable angina, and angina pectoris to those of controls measured by enzyme-linked immunosorbent assay kits; (a) substance P, (b) neuropeptide Y, and (c) *N*-terminal pro-B-type natriuretic peptide. (*: *P* value < 0.05).

NPY was the only marker candidate for UA patients. The number of patients having hypertensive was 2- or 3-fold higher than non-patients.

Comparison of Biomarker Candidates. Figure 3 showed a correlation between concentrations of each peptide biomarker candidate. CTn is the well-known biomarker for diagnosis of AMI patient. The NT-proBNP only showed a correlation with the cTn (R^2 = 0.8976, P < 0.0001, Figure 3(d)). NPY or SubP didn't show correlation with cTn and NT-proBNP (Figure 3(b) and (c)). The NPY and SubP have a correlation each other (R^2 = –0.4567, P = 0.0112, Figure 3(a)). We also tested the correlation between cTn and the others peptides in serum samples of acute myocardial infarction patients. From these results, we can assume that the reaction mechanism of NPY and SubP release into serum might be different from that of NT-proBNP and cTn. NT-proBNP and cTn are already known as predictors in acute coronary syndrome, combining them for an estimation of long-term prognosis.[39] The study on production and release mechanism of SubP and NPY could explain action mechanism of marker candidates in AMI progress and would give a therapeutic hint.

To evaluate the performance of the marker candidates for diagnosis of AMI, ROC curve was calculated. The area under the curve (AUC) is a popular indicator of test accuracy.[40] The AUCs with 95% confidence intervals (CI) were higher than 0.80 among three biomarker candidates. Table 4 showed the cut-off values, AUCs, specificity, and sensitivity calculated by ROC plot for AMI patients compared to healthy controls. The suggested cut-off values for AMI patients were 53.58 (SubP), 525 (NPY), and 246.2 (NT-proBNP) pg/mL. The specificity (90%) and sensitivity (87%) of NPY were the highest among three marker candidates. SubP gave higher specificity (83%) and sensitivity (80%) than NT-proBNP which was reported as an early marker candidate for the diagnosis of AMI patients in many groups.[10,21,27] From above results, we suggested SubP and NPY as early diagnostic biomarkers for AMI patients. The combination of two peptides analysis will give a chance to find for AMI patients in early stage. We calculated ROC curves of AMI patients compared to non-AMI patients and controls. The results were similar to the comparison of between AMI and healthy controls. The sensitivity and specificity were 80%, 75% for SubP, 86.7%, 82.6% for NPY, and 60%, 78.2% for NT-proBNP. It indicates that the sensitivity and specificity are better SubP and NPY of between AMI and non-AMI patients than those

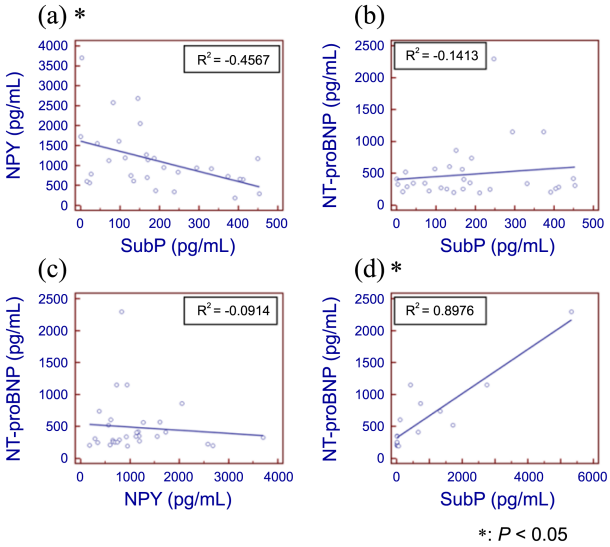

Figure 3. Correlation between marker candidate concentrations (substance P, neuropeptide Y, *N*-terminal pro-B-type natriuretic peptide, and cardiac troponin) in serum samples of acute myocardial infarction patient; (a) substance P and neuropeptide Y (*P* value: 0.0112, R^2 value: –0.4567), (b) substance *P* and *N*-terminal pro-B-type natriuretic peptide (*P* value: 0.4565, R^2 value: 0.1413), (c) neuropeptide Y and *N*-terminal pro-B-type natriuretic peptide (P value: 0.6308, R^2 value: –0.0914), and (d) *N*-terminal pro-B-type natriuretic peptide and cardiac troponin (*P* value: < 0.0001, R^2 value: 0.8976).

Table 4. Specificity, sensitivity, and the suggested cut-off value for the diagnosis of acute myocardial infarction using receiver operating characteristic curve

Parameters	SubP	NPY	NT-proBNP
Cut-off value (pg/mL)	53.58	525	246.2
AUC[a,b]	0.80 ± 0.062	0.87 ± 0.056	0.80 ± 0.057
Specificity	83%	90%	69%
Sensitivity (95% CI)	80% (61-92)	87% (69-96)	80% (61-92)

[a]AUC ± standard error. [b]All $P < 0.05$ when comparing AUC

of NT-proBNP.

Conclusion

The results obtained in this work demonstrate that SubP and NPY could possibly be used as biomarkers for AMI diagnosis together with NT-proBNP and cTn in the early stage of AMI for Koreans. NPY showed the highest specificity (90%) and sensitivity (87%). Simultaneous detection of SubP and NPY could be utilized as a valuable biomarker for early diagnosis of AMI patients differentiated from UA. Until now, the sensitivity of the commercially available ELISA kit for SubP was not sufficient yet (LOQ = 31.5 pg/mL). In further studies, we plan to develop an ELISA method with higher sensitivity for the measurement of marker candidates that are present in the serum at low concentrations.

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