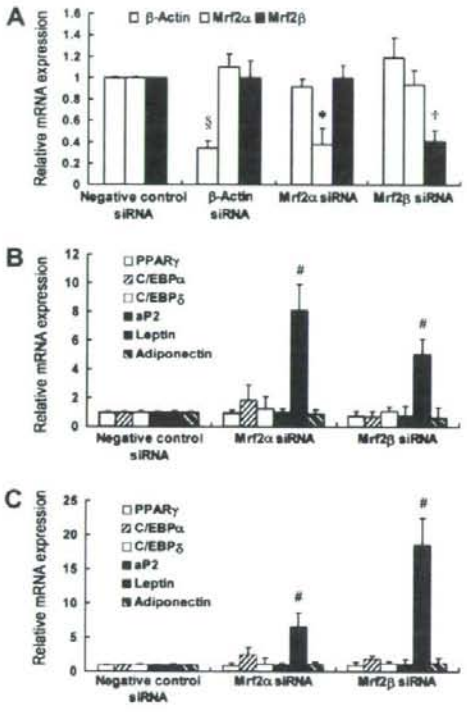

Fig. 3. Effects of down-regulation of Mrf2α and Mrf2β mRNA expressions using siRNA on adipogenesis transcription factors and adipokines. The fully differentiated 3T3-L1 adipocytes were transfected with 30 nM siRNA and the complete growth medium was added 4 h after transfection. (A) Mature adipocytes were transfected with negative control siRNA (negative control), β-actin siRNA (positive control), Mrf2α siRNA, and Mrf2β siRNA. After 24 h total RNA was extracted. Levels of β-actin mRNA, Mrf2α mRNA, and Mrf2β mRNA were measured using quantitative real-time PCR. $^{\S}P < 0.05$ vs. β-actin, $^{*}P < 0.05$ vs. Mrf2α, and $^{\dagger}P < 0.05$ vs. Mrf2β in cells transfected by negative control siRNA ($n = 4$). (B) Negative control siRNA, Mrf2α, and Mrf2β siRNA were transfected into mature adipocytes and RNA was extracted 24 h after transfection. (C) Negative control siRNA, Mrf2α, and Mrf2β siRNA were transfected into mature adipocytes and RNA was extracted 48 h after transfection. The mRNA expressions of PPARγ2, C/EBPα, C/EBPδ, aP2, leptin, and adiponectin were measured using quantitative real-time PCR. Rps3 mRNA expression was used as an internal control. $^{\#}P < 0.05$ vs. leptin mRNA expressions in cells transfected with negative control siRNA ($n = 4$).

increased by 5.0 ± 1.0-fold at 24 h (Fig. 3B) and by 18.6 ± 1.4-fold at 48 h (Fig. 3C). Although C/EBPα mRNA was modestly increased by approximately 2-fold after Mrf2α siRNA was transfected, it did not reach statistical significance. Rps3 mRNA expression was stable throughout the experiments.

Discussion

Many transcription factor families are altered during the various stages of adipocyte differentiation and play pivotal roles in adipogenesis. These include C/EBP, PPAR, sterol regulatory element-binding protein (SREBP), cAMP response element-binding protein (CREB), Kruppel-like factor (KLF), and nuclear factor of activated T cell (NFAT) family. They form complex transcription regulation network. These factors affect each other and regulate the expression of adipokine positively and negatively [7–11].

Mrf2 is a DNA-binding transcriptional regulator and was originally recognized to play roles in differentiation of smooth muscle cells [1]. In this study 3T3-L1 cells were used as a model of adipocyte differentiation through exposure to appropriate hormonal inducers. Because specific antibody to Mrf2α is not yet available, our research was focused on mRNA expression. Mrf2α and Mrf2β mRNA expressions were markedly up-regulated during the early stage of differentiation and high expression levels were maintained in mature adipocytes, suggesting that Mrf2 might be involved in regulation of adipocyte differentiation and might be a new member of the adipogenic transcription network.

Adipose tissue is involved in energy metabolism, inflammation, and endocrine system. They exhibit high reactivity to stimulations by insulin, glucocorticoids, and some inflammatory factors. The defect in insulin reactivity contributes to metabolic syndrome [12]. Like C/EBP and PPAR family [8,13,14] Mrf2α mRNA was regulated by insulin and dexamethasone in mature 3T3-L1 cells and insulin decreased Mrf2α and Mrf2β mRNA expressions. Dexamethasone increased Mrf2α mRNA expression and decreased Mrf2β mRNA expression. Meanwhile Mrf2 mRNA expression was up-regulated by insulin and dexamethasone in preadipocytes. These results suggest that Mrf2 is a potent regulator of insulin and glucocorticoid signaling pathways. Only minor changes of Mrf2 mRNA were found in response to TNFα. For other inflammatory cytokines virtually no changes of Mrf2 mRNA expression were found in response to IL-1 and IL-6 (data not shown).

To clarify which genes were regulated by Mrf2, Mrf2 expression was decreased by using siRNA interference strategy. It was found that leptin mRNA expression was markedly increased when Mrf2α and Mrf2β gene expressions were silenced in fully differentiated 3T3-L1 cells. No changes were found on the mRNA expressions of fatty acid-binding protein aP2 (a marker of adipocyte differentiation), PPARγ2, and adiponectin. The expression of C/EBPα mRNA was modestly increased when Mrf2α gene expressions were silenced. These results suggest that Mrf2α may negatively regulate leptin expression as a transcription repressor. Consistent with these findings Yamakawa et al. also reported recently that Mrf2 stimulates adipogenesis in mouse embryo fibroblasts and 3T3-L1 cells [15].

Homozygous mice deficient in C/EBPα, PPARγ2, SREBP-1c, CREB or Mrf2 exhibit high rates of neonatal mortality. All of heterozygous deficient mice of these adipogenic transcription factors were very lean and showed markedly reduced weight of white adipose tissue and loss

of accumulation of triglyceride in adipocytes. They are completely protected from body weight gain induced by high-fat diet. These knockout mice have different characteristics in terms of energy metabolism [3,16–21]. C/EBPα$^{+/-}$ and SREBP-1c$^{+/-}$ mice are lipoatrophic. They are accompanied by severe insulin resistance, leading to hyperinsulinemia, hyperglycemia, enlarged fatty liver, and diabetes mellitus from birth. Levels of leptin were decreased [16–19]. CREB$^{+/-}$ and PPARγ$^{+/-}$ mice have opposite phenotypes. They exhibit increased insulin sensitivity, glucose tolerance, increased expression and sensitivity to leptin and adiponectin, and normal liver [9,20,21]. Mrf2$^{+/-}$ mice are similar to CREB$^{+/-}$ and PPARγ$^{+/-}$, and they showed high insulin sensitivity, and normal liver weight [3]. It is unclear whether plasma leptin levels are altered in Mrf2$^{+/-}$ mice. Because leptin is synthesized mainly in adipose tissue, it is speculated that leptin would be increased in accordance with our data on Mrf2 siRNA experiment.

Leptin regulates energy balance, metabolism, and neuroendocrine response to altered nutrition. Replacing leptin in leptin deficient (ob/ob) mice and in humans leads to decrease of body weight and depletion of lipid in adipose tissue, liver, and other tissues. Leptin treatment also improves insulin sensitivity. The metabolic changes can be explained by the actions of leptin in decreasing food intake and increasing fatty acid oxidation, and energy expenditure [18,22,23]. Thus, increased insulin sensitivity and loss of fat in Mrf2α knockout mice could be explained at least partly by up-regulation of leptin. As leptin treatment can become a potent therapeutic strategy of metabolic syndrome, it is possible that Mrf2α will be one of the important therapeutic targets to elevate leptin expression.

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Risk Stratification of Chronic Heart Failure Patients by Multiple Biomarkers

— Implications of BNP, H-FABP, and PTX3 —

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Background B-type natriuretic peptide (BNP), heart-type fatty acid-binding protein (H-FABP), and pentraxin 3 (PTX3) each predict adverse cardiac events in chronic heart failure (CHF) patients. For prognostic evaluation from different aspects, the utility of combined measurement of the 3 biomarkers in patients with CHF was examined in the present study.

Methods and Results Levels of BNP (associated with left ventricular dysfunction, positive if >200pg/ml), H-FABP (marker of myocardial damage, positive if >4.1 ng/ml), and PTX3 (marker of inflammation, positive if >4.0ng/ml) were measured in 164 consecutive CHF patients, and patients were prospectively followed with endpoints of cardiac death or rehospitalization. When patients were categorized on the basis of the number of elevated biomarkers, patients with 1, 2, and 3 elevated biomarkers had a 5.4-fold (not significant), 11.2-old ($p<0.05$), and 34.6-fold increase ($p<0.01$), respectively, in the risk of adverse cardiac events compared with those without elevated biomarkers. Kaplan-Meier analysis revealed that patients with 3 elevated biomarkers had a significantly higher cardiac event rate than patients with a lower number of elevated biomarkers.

Conclusion The combination of these 3 biomarkers could reliably risk-stratify CHF patients for prediction of cardiac events. (*Circ J* 2008; **72:** 1800–1805)

Key Words: Biomarkers; Chronic heart failure; Risk stratification

Chronic heart failure (CHF) is still a major cause of death and hospitalization, and has a poor prognosis despite the significant reduction in mortality achieved in clinical trials.[1–3] Therefore, the prognostic evaluation and risk stratification of CHF patients continues to increase in importance and involves a complex assessment of multiple interacting variables.

Several new cardiac biomarkers have emerged as strong predictors of risk among CHF patients. Importantly, these biomarkers are mainly divided into 3 different pathophysiological aspects: (1) neurohormonal markers that reveal pressure and/or volume overload, (2) markers of myocardial damage, and (3) markers of inflammation. B-type natriuretic peptide (BNP) is secreted from the ventricles by mechanical overload and is the most established marker of neurohormonal factors.[4] Heart-type fatty acid-binding protein (H-FABP) is a novel marker of ongoing myocardial cell injury,[5,6] and pentraxin 3 (PTX3) is a novel marker of inflammation.[7] These 3 biomarkers have been used for predicting cardiac events in CHF patients,[4–12] but the incremental usefulness of the combination of these 3 biomarkers has not been previously examined in patients with CHF. Recently, a multi-axis framework has been proposed in order to more completely appreciate the mechanisms of cardiovascular diseases. Thus, we hypothesized that the combination of 3 biomarkers would provide complementary information and stratify risk more effectively among patients with CHF.

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Methods

Study Design

We prospectively studied 164 consecutive patients (92 men, 72 women; mean age, 68±14 years) who were admitted to the Yamagata University Hospital from April 1996 to February 2005 for the treatment of worsening CHF, for diagnosis and pathological investigation of heart failure, or for therapeutic evaluation of heart failure. The diagnosis of CHF was based on history of dyspnea and symptomatic exercise intolerance with signs of pulmonary congestion or peripheral edema or documentation of left ventricular enlargement or dysfunction by chest X-ray, echocardiography or radionuclide ventriculography. Baseline characteristics of the study subjects are listed in Table 1. The diagnoses of hypertension, diabetes, and hyperlipidemia were obtained from medical records or patient history of currently or previously received medical therapy. Exclusion criteria in this study were those with clinical or electrocardiographic evidence suggestive of acute coronary syndrome within the 3 months preceding admission, those with renal insufficiency characterized by a serum creatinine concentration

>1.5 mg/dl, those with inflammatory disease and any documented inflammatory illness such as arthritis, connective tissue disease, active hepatic disease, pulmonary disease, or any malignancy. Informed consent was given by all patients before participation in this study, and the protocol was approved by the institution's Human Investigations Committee.

Blood samples were obtained on admission for measurement of plasma BNP, serum H-FABP, and plasma PTX3 levels. The optimal cut-off values for the 3 biomarkers were determined as those with the largest sum of sensitivity plus specificity on each of the receiver-operating characteristic (ROC) curves. Cut-off values of BNP (200 pg/ml), H-FABP (4.1 ng/ml), and PTX3 (4.0 ng/ml) were determined by ROC curves as shown in Fig 1. Patients were categorized into 4 groups on the basis of the number of elevated biomarkers (score 0–3). Glomerular filtration rate (GFR) was estimated from the modification of diet in renal disease equation modified by a Japanese coefficient[13]

Transthoracic echocardiography was performed by experienced echocardiologists without knowledge of the biochemical data, using an ultrasound instrument (Hewlett Packard SONOS 7500, Palo Alto, CA, USA) equipped with a sector transducer (carrier frequency of 2.5 or 3.75 MHz) within 1 week after admission.

Endpoints and Follow-up

No patients were lost to follow-up (mean follow-up 679± 438 days) after admission. Events were centrally adjudicated using medical records, autopsy reports and death certificates. The endpoints, which were judged independently by researchers, were (1) cardiac death, defined as death from worsening heart failure or sudden cardiac death, and (2) worsening heart failure requiring readmission. Sudden cardiac death was defined as death without definite premonitory symptoms or signs and was established by the attending physician.

Assays of BNP, H-FABP, and PTX3

BNP The venous blood samples were transferred to chilled tubes containing 4.5 mg of ethylenediaminetetraacetic acid disodium salt and aprotinin (500 U/ml), and immediately centrifuged at 1,000 G for 15 min at 4°C to examine plasma biomarkers. The clarified plasma samples were frozen, stored at −70°C and thawed just before assay. Plasma BNP levels were measured using a commercially available specific radioimmunoassay for human BNP (Shiono RIA BNP assay kit, Shionogi Co Ltd, Tokyo, Japan)[5]

H-FABP The venous blood samples were immediately centrifuged at 2,500 G for 15 min at 4°C to measure serum H-FABP levels. The clarified serum samples were frozen, stored at −70°C, and thawed just before assay. H-FABP levels were measured using a 2-step sandwich enzyme-linked immunosorbent assay (ELISA) kit (MARKIT-M H-FABP, Dainippon Pharmaceutical Co Ltd, Tokyo, Japan) as previously reported[6]

PTX3 Plasma PTX3 levels were measured using the ELISA kit (Perseus Proteomics Inc, Tokyo, Japan) as previously reported[7]

The analytical range of these kits was 4.0–2,000 pg/ml for BNP assay, 1.1–250 ng/ml for H-FABP assay, and 0.1–20 ng/ml for PTX3 assay.

Statistical Analysis

Results are presented as mean±standard deviation (SD) values for continuous variables and as the percentage of total patients for categorical variables. Skewed variables are presented as median and interquartile range. Unpaired Student's t-test and the chi-square test were used for comparisons between 2 groups of continuous and categorical variables, respectively. If data were not distributed normally, the Mann-Whitney U-test was used. Comparison of data

Table 1 Clinical Characteristic of 164 Patients With CHF

Age (years)	*68±14*
Gender (M/F)	*92/72*
NYHA functional class (I/II/III/IV)	*36/68/47/13*
Hypertension	*81 (49%)*
Hyperlipidemia	*35 (21%)*
Diabetes mellitus	*40 (24%)*
Current smoking	*29 (18%)*
Etiology of heart failure	
Dilated cardiomyopathy	*44 (27%)*
Valvular heart disease	*44 (27%)*
IHD	*38 (23%)*
Others	*38 (23%)*
Echocardiography	
LVEDD (mm)	*54.3±9.4*
LVEF (%)	*49.6±19.3*
Laboratory data	
eGFR (ml·min^{-1}·1.73 m^{-2})	*64.1±20.1*
Uric acid (mg/dl)	*6.2±2.0*
BNP (pg/ml)	*277 (88–723)*
H-FABP (ng/ml)	*4.3 (3.0–6.9)*
PTX3 (ng/ml)	*3.8 (2.2–6.5)*
No. of elevated biomarkers	*1.6±1.1*
Cardiac events	*49 (30%)*
Rehospitalization	*31 (19%)*
Cardiac death	*18 (11%)*
Pharmacotherapy	
ACEIs or ARBs	*105 (64%)*
β-blockers	*61 (37%)*
Calcuim-channel blockers	*33 (20%)*
Diuretics	*116 (71%)*
Statins	*30 (18%)*

Skewed data are reported as median (interquartile range).
CHF, chronic heart failure; NYHA, New York Heart Association; IHD, ischemic heart disease; LVEDD, left ventricular dimension at end-diastole; LVEF, left ventricular ejection fraction; eGFR, estimated glomerular filtration rate; BNP, B-type natriuretic peptide; H-FABP, heart-type fatty acid-binding protein; PTX3, pentraxin 3; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker.

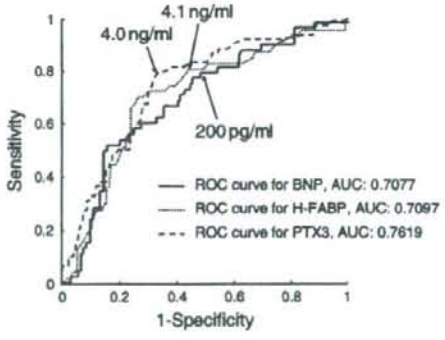

Fig 1. Receiver-operating characteristic (ROC) curve analysis of B-type natriuretic peptide (BNP), heart-type fatty acid-binding protein (H-FABP), and pentraxin 3 (PTX3). AUC, area under the ROC curve.

Table 2 Comparison of the Clinical Characteristics of Patients With and Without Cardiac Events

	Event-free (n=115)	Cardiac event (n=49)	p value
Age (years)	67±14	71±12	0.0470
Gender (M/F)	65/50	27/22	NS
Hypertension	61 (53%)	20 (41%)	NS
Hyperlipidemia	22 (19%)	13 (26%)	NS
Diabetes mellitus	28 (24%)	12 (24%)	NS
Current smoking	20 (17%)	9 (18%)	NS
NYHA functional class (I/II/III/IV)	33/50/24/8	3/18/23/5	0.0007
Etiology of heart failure			
IHD	29 (25%)	9 (18%)	NS
Non-IHD	86 (75%)	40 (82%)	NS
Echocardiography			
LVEDD (mm)	53.4±8.6	56.6±10.9	NS
LVEF (%)	51.3±17.0	45.7±17.8	NS
Laboratory data			
eGFR (ml·min^{-1}·1.73 m^{-2})	67.4±19.6	59.0±20.2	0.0131
Uric acid (mg/dl)	6.0±2.1	6.7±1.8	0.0253
BNP (pg/ml)	198 (66–507)	707 (227–1,234)	0.0028
H-FABP (ng/ml)	3.8 (2.8–5.3)	6.1 (4.3–8.9)	0.0054
PTX3 (ng/ml)	3.2 (2.0–5.5)	6.0 (4.3–9.3)	<0.0001
No. of elevated biomarkers	1.24±1.01	2.39±0.81	<0.0001

Skewed data are reported as median (interquartile range).
Abbreviations as in Table 1.

Table 3 Results of Univariate Cox Proportional Hazard Analysis

	Hazard ratio	95%CI	p value
Age, per 5-year increase	1.19	1.04–1.36	0.0107
Gender (M/F)	1.04	0.59–1.83	NS
Hypertension	0.73	0.41–1.29	NS
Hyperlipidemia	1.83	0.97–3.46	NS
Diabetes mellitus	1.03	0.54–1.98	NS
Current smoking	1.07	0.05–2.20	NS
NYHA I/II or III/IV	3.28	1.80–5.95	<0.0001
IHD or non-IHD	1.75	0.84–3.61	NS
LVEDD, 9.5-mm increase	1.32	0.99–1.77	NS
LVEF, 17.4% increase	0.77	0.57–1.02	NS
eGFR per 20.1-ml increase	0.64	0.46–0.89	0.0078
Uric acid, 2.0-mg/dl increase	1.30	1.01–1.66	0.0391
BNP, 758-pg/ml increase	1.40	1.00–2.13	0.0010
H-FABP, 5.5-ng/ml increase	1.66	1.32–2.09	<0.0001
PTX3, 4.7-ng/ml increase	1.42	1.20–1.70	<0.0001

CI, confidence interval. Other abbreviations as in Table 1.

among 4 groups categorized on the basis of the number of elevated biomarkers was performed by the Kruskal-Wallis test. A Cox proportional hazard analysis was performed to evaluate the associations between cardiac events and measurements. The cardiac event-free curve was computed according to the Kaplan-Meier method and compared by the log-rank test. The ROC curves were constructed to illustrate various cut-off values of BNP, H-FABP and PTX3 for predicting cardiac events at 36 months and to determine optimal sensitivity and specificity.[14] All p-values reported are 2-sided, and $p<0.05$ was considered significant. Statistical analysis was performed with a standard statistical program package (StatView, version 5.0, SAS Institute Inc, Cary, NC, USA).

Result

Patient Characteristics

The baseline characteristics of 164 heart failure patients are shown in Table 1. The mean age of study subjects was 68±14 years old, 56% of patients were men and 36% were in New York Heart Association (NYHA) functional class III or IV. The etiologies of heart failure were dilated cardiomyopathy in 27% and ischemic heart disease in 23% (Table 1). A simple scoring system based upon the number of elevated 3 biomarkers was 1.6±1.1. Correlations of these markers were very weak (BNP and H-FABP: R=0.342, $p<0.0001$; BNP and PTX3: R=0.235, p=0.0025; H-FABP and PTX3: R=0.216, p=0.0054), suggesting that these markers reflect different features of the pathophysiologic process of heart failure.

Clinical Outcomes

All patients were followed-up completely. There were 49 cardiac events (30%), comprising 18 cardiac deaths and 31 re-hospitalizations for worsening heart failure during the follow-up period.

As shown in Table 2, patients with cardiac events were older, and had more severe NYHA functional class than those without cardiac events. Furthermore, patients with

Table 4 Results of Multivariate Cox Proportional Hazard Analysis

	Hazard ratio	95% CI	p value
Age, 5-year increase	1.08	0.96–1.23	NS
NYHA I/II vs III/IV	1.69	0.79–3.59	NS
eGFR, 20.1-ml increase	0.96	0.67–1.40	NS
Uric acid, 2.0-mg/dl increase	1.14	0.86–1.50	NS
BNP, 758-pg/ml increase	1.05	1.00–1.08	NS
H-FABP, 5.5-ng/ml increase	1.29	0.91–1.83	NS
PTX3, 4.7-ng/ml increase	1.24	1.01–1.53	0.0458

Abbreviations as in Tables 1,3.

Table 5 Clinical Characteristics of the 4 Groups of CHF Patients

No. of elevated biomarkers	Score 0 (n=33)	Score 1 (n=46)	Score 2 (n=41)	Score 3 (n=44)
Age (years)	60±10	69±11**	66±18*	75±10**,#,††
Gender (M/F)	21/12	24/22	23/18	24/20
Hypertension	17 (52%)	22 (48%)	23 (56%)	19 (43%)
Hyperlipidemia	7 (21%)	12 (26%)	5 (12%)	11 (25%)
Diabetes mellitus	7 (21%)	14 (30%)	12 (29%)	7 (16%)
Current smoking	7 (21%)	8 (17%)	8 (20%)	6 (14%)
NYHA functional class				
I/II	33 (100%)	36 (78%)	21 (51%)	14 (32%)§
III/IV	0 (0%)	10 (22%)	20 (49%)	30 (68%)§
Etiology of heart failure				
IHD	6 (18%)	11 (24%)	10 (24%)	11 (25%)
Non-IHD	27 (82%)	35 (76%)	31 (76%)	33 (75%)
Echocardiography				
LVEDD (mm)	52.8±8.5	53.5±9.2	54.3±7.9	56.3±11.5
LVEF (%)	63.0±12.8	50.0±15.0**	48.5±17.6**	40.6±2.5**,#,†
Laboratory data				
eGFR (ml·min^{-1}·1.73m^{-2})	76.1±18.8	67.1±18.5*	65.5±20.0*	53.7±17.9**,#,††
Uric acid (mg/dl)	5.9±2.1	6.1±2.0	5.8±1.8	6.9±2.0*,†
BNP (pg/ml)	68 (38–112)	138 (56–294)	410 (213–736)**,##	1,035 (555–1,685)**,#,††
H-FABP (ng/ml)	2.8 (2.1–3.4)	4.1 (3.1–4.8)	4.5 (2.9–6.8)*	7.6 (6.1–11.0)**,#,††
PTX3 (ng/ml)	2.0 (1.7–2.5)	2.8 (2.0–3.7)	5.0 (3.2–7.0)**,#	6.8 (5.1–10.9)**,#,††

Skewed data are reported as median (interquartile range).
**p<0.05, **p<0.01 vs score 0; #p<0.05, ##p<0.01 vs score 1; †p<0.05, ††p<0.01 vs score 2; §p<0.01 by chi-square test.*
Abbreviations as in Table 1.

cardiac events showed higher levels of uric acid, BNP, H-FABP, PTX3, lower estimated GFR (eGFR), and higher score based upon the number of elevated biomarkers compared with those without cardiac events, whereas other parameters, including gender and numbers of patients who had hypertension, diabetes mellitus, hyperlipidemia, or were currently smoking, were not significantly different between patients with and without cardiac events.

The univariate Cox proportional hazard analysis to predict cardiac events is shown in Table 3. Age, NYHA classification, eGFR, uric acid, BNP, H-FABP, and PTX3 were related significantly to cardiac events. Those variables with p-values less than 0.05 were entered into the multivariate Cox proportional hazard regression analysis (Table 4), and only PTX3 was an independent predictor of future cardiac events.

Classification by Number of Elevated Biomarkers

Patients were categorized into 4 groups (score 0–3) on the basis of the number of elevated biomarkers (Table 5). Cut-off values of BNP (200 pg/ml), H-FABP (4.1 ng/ml), and PTX3 (4.0 ng/ml) were determined by ROC curves, as shown in Fig 1. The number of patients over each cut-off value determined by the ROC curves was 95 (58%) patients for BNP >200 pg/ml, 84 (51%) for H-FABP >4.1 ng/ml, and 81 (49%) for PTX3 >4.0 ng/ml.

Patients with score 3 were older, and had a more severe NYHA functional class, lower left ventricular ejection fraction, lower eGFR, and higher levels of uric acid, BNP, H-FABP, and PTX3 compared with those with score 0–2 (Table 5). Furthermore, patients with score 3 had significantly higher rates of rehospitalization and cardiac death than those with score 0–2 ($p<0.0001$; Fig 2A). Other parameters, including gender and etiology of CHF, were not significantly different among the 4 groups. In addition, there was no difference among the 4 groups in the numbers of patients who had hypertension, diabetes mellitus, hyperlipidemia, or were currently smoking.

Kaplan-Meier analysis demonstrated that patients with score 3 had significantly higher cardiac event rates than patients with score 0–2 (Fig 2B). These results suggest that when the number of elevated biomarkers is high, prognosis is poor and intense follow-up after discharge with chest X-ray, echocardiography, and blood examination is recommended.

Risk Stratification by Number of Elevated Biomarkers

Prognostic results of the univariate Cox proportional hazard analysis to predict cardiac events are shown in Fig 3: patients with score 1, 2, and 3 had a 5.4-fold, 11.2-fold

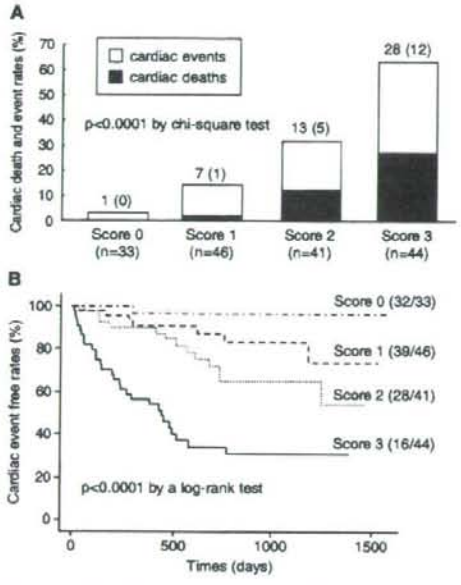

Fig 2. Cardiac mortality and all cardiac events. (A) Patients with score 3 had the highest rates of rehospitalization and cardiac death among the 4 scoring groups. **p<0.001 by chi-square test. (B) Kaplan-Meier analysis in chronic heart failure patients stratified into 4 groups based on the number of elevated biomarkers. Patients with score 3 had significantly higher rates of cardiac events than patients with score 0–2.

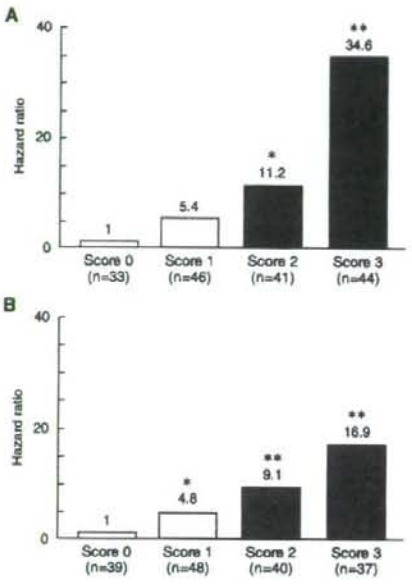

Fig 3. Hazard ratios to predict cardiac events. Univariate Cox proportional hazard analysis demonstrated that patients with score 3 were associated with the highest risk for cardiac events among 4 groups. Patients were categorized by B-type natriuretic peptide (BNP), heart-type fatty acid-binding protein (H-FABP), and pentraxin 3 (A) and by BNP, H-FABP, and New York Heart Association classification (B). *p<0.05 and **p<0.01 vs patients with score 0.

(p<0.05), and 34.6-fold increase (p<0.01), respectively, in the risk of adverse cardiac events compared with those with score 0 (Fig 3A). When patients were categorized by BNP, H-FABP, and NYHA classification, patients with score 1, 2, and 3 had a 4.8-fold, 9.1-fold, and 16.9-fold increase, respectively, in the risk of adverse cardiac events compared with score 0 (Fig 3B). In addition, an increase of one score had a hazard ratio of 2.801 in the combination of BNP, H-HAFBP, and PTX3, and hazard ratio of 2.142 in the combination of BNP, H-FABP, and NYHA classification. These data clearly demonstrate that the combination of BNP, H-FABP, and PTX3 can stratify risk of CHF more effectively than the combination of BNP, H-FABP and NYHA class.

Discussion

We have shown that the number of elevated biomarkers (BNP, H-FABP, and PTX3) was significantly higher in patients with cardiac events than in those without cardiac events. Because CHF is a major public health problem[1–3] it is necessary to grade the severity of CHF patients. This study examined whether the combination of BNP, H-FABP, and PTX3 provides valuable information for risk stratification in patients with CHF. Univariate Cox proportional hazard analysis demonstrated that patients with 3 elevated biomarkers were associated with the highest risk (34.6-fold) for cardiac events compared with patients with a lesser number of elevated biomarkers. Furthermore, Kaplan-Meier analysis demonstrated that cardiac events occurred most frequently in patients with 3 elevated biomarkers compared with patients with less than 3 elevated biomarkers. These results suggest that the combination of these 3 biomarkers could improve risk stratification for the prediction of cardiac events in CHF patients.

Because CHF is accompanied by a variety of pathological changes that trigger disease progression, a multi-axis framework has been proposed in order to more effectively appreciate the pathophysiology of CHF[15,16] Therefore, the combination of multiple biomarkers, which is a novel method of risk stratification of CHF patients, may provide helpful information for understanding different aspects of the interrelated pathophysiological processes of CHF.

We selected 3 different pathogenic features (neurohormonal markers, markers of myocardial damage, and markers of inflammation) on the basis of previous clinical studies. BNP is secreted from the ventricles by mechanical overload and is a well-established prognostic factor in CHF patients[4,8] H-FABP is abundant in the cytosol of cardiomyocytes and is released into the circulation when the cell surface membrane is injured[17–19] H-FABP levels are increased in patients with advanced CHF because of leakage of cytosolic proteins from cardiomyocytes affected by the ongoing myocardial damage[5] We previously reported that H-FABP was more sensitive than troponin T, a myofibrillar component, for detecting ongoing myocardial damage[6]

Recently it was reported that inflammatory markers, such as high-sensitivity C-reactive protein (hs-CRP) and tumor necrosis factor-alpha (TNF-α), were related to decreasing functional status and provide important prognostic information about the morbidity and mortality of CHF patients[20,21] PTX3 is in the pentraxin superfamily and a newly discovered marker of the acute-phase inflammatory response[22] We previously showed that PTX3 was superior to hs-CRP and TNF-α in predicting cardiac events of CHF patients[7] so in the present study we examined the possibility of combining the measurement of BNP, H-FABP, and PTX3 to reflect different aspects of CHF. Improved risk stratification of CHF patients may depend on the discovery of new biomarkers. In addition, it is necessary in a future study to find treatments to reduce these biomarkers and improve clinical outcomes.

Conclusions

Our data suggest that measuring the combination of BNP, H-FABP, and PTX3 is highly reliable method for risk stratification of patients hospitalized for CHF. It is necessary to examine in a future study whether this approach allows clinicians to improve the management of CHF patients.

Acknowledgments

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Relation of Serum Heat Shock Protein 60 Level to Severity and Prognosis in Chronic Heart Failure Secondary to Ischemic or Idiopathic Dilated Cardiomyopathy

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Heat shock protein (HSP) 60 is induced by a variety of stressors, including oxidative stress and inflammation, and it plays a protective role against stress-induced cardiomyocyte injury. Recently, it has been reported that HSP 60 exists in the circulation. Chronic heart failure (CHF) is characterized by systemic abnormalities, and the myocardium is exposed to various stressors. However, the clinical significance of serum HSP 60 has not been examined in CHF. Therefore, the purpose of this study was to examine whether HSP 60 is correlated with the severity of CHF and whether HSP 60 can predict clinical outcomes in patients with CHF. Serum HSP 60 levels were measured in 112 patients with CHF and 62 control subjects. Serum HSP 60 levels were higher in patients with CHF than in control subjects and increased with advancing New York Heart Association functional class. There were 37 cardiac events during a mean follow-up period of 569 ± 476 days (range 17 to 1,986). Serum HSP 60 levels were higher in patients with cardiac events than in event-free patients. Patients were divided into 4 groups on the basis of HSP 60 level. Cox proportional-hazards regression analysis and Kaplan-Meier analysis revealed that the fourth quartile was associated with the greatest risk for cardiac events. In conclusion, serum HSP 60 level was related to the severity of CHF and associated with a high risk for adverse cardiac events in patients CHF. © 2008 Elsevier Inc. All rights reserved. (Am J Cardiol 2008;102:606–610)

Heat shock protein (HSP) is constitutively expressed, and its expression is upregulated by a variety of stressors, including infection, anoxia, oxidative stress, and inflammation. It has been reported that HSP 60 has a protective function against stress-induced injury.[1–4] HSP 60 is primarily a mitochondrial protein that is expressed at high levels in the normal cells and is essential for the maintenance of normal cellular function.[5] Several studies have reported that the overexpression of HSP 60 prevents apoptotic cell death induced by ischemia in myocardial cells in vivo and in vitro.[6–8] Recent studies have demonstrated that HSP 60 has a cytokine-like activity and induces tumor necrosis factor–α and interleukin-6 production, suggesting an association between HSP 60 and inflammatory and infectious responses.[9,10] In addition, it has been reported that HSP 60 exists in the circulation of healthy subjects, and serum HSP 60 levels are positively associated with interleukin-6 and an important proinflammatory cytokine.[11] Chronic heart failure (CHF) is characterized by systemic abnormalities, and the myocardium is continually exposed to various stressors, such as anoxia, ischemia, inflammatory, and free radicals.[12] Although Knowlton et al[13] showed an increase in the HSP 60 content of the human heart in the end stages of ischemia- and dilation-elicited cardiomyopathies, the clinical significance of serum HSP 60 levels has not been examined in CHF. Therefore, the purpose of this study was to examine whether serum levels of HSP 60 are correlated with the severity of CHF and whether serum HSP 60 can predict clinical outcomes in patients with CHF.

Methods

From October 1998 and November 2005, we prospectively studied 112 consecutive patients with CHF secondary to ischemic or idiopathic dilated cardiomyopathy (74 men and 38 women) who had been admitted for the treatment of worsening CHF, for diagnostic and pathophysiologic investigations, or for therapeutic evaluations of CHF. The diagnosis of CHF was made by 2 senior cardiologists using the generally accepted Framingham criteria and information including a history of dyspnea and symptomatic exercise intolerance, with signs of pulmonary congestion or peripheral edema or the presence of moist rales on auscultation or documentation of left ventricular enlargement or dysfunction by chest x-ray or echocardiography.[14] The diagnosis of dilated cardiomyopathy was based on the definition of the World Health Organization and International Society and Federation of Cardiology task force.[15] Informed consent was obtained from all patients before participation in the

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Table 1
Clinical characteristics of 112 patients with chronic heart failure and 62 control subjects

Variable	Control Subjects (n = 62)	Patients With CHF (n = 112)	p Value
Age (yrs)	67 ± 10	67 ± 13	0.8285
Men/women	37/25	74/38	0.4021
NYHA functional class (I/II/III/IV)	—	22/44/33/13	—
Hypertension	30 (49%)	58 (52%)	0.6676
Diabetes mellitus	9 (15%)	30 (27%)	0.0931
Hyperlipidemia	10 (16%)	29 (26%)	0.0879
Current smokers	14 (23%)	29 (26%)	0.7535
Dilated cardiomyopathy	—	64 (32%)	—
Ischemic heart disease	—	48 (24%)	—
Blood examination			
Sodium (mEq/L)	143 ± 2.0	140 ± 3.3	0.0898
Creatinine (mg/dl)	0.75 ± 0.19	0.99 ± 0.44	0.0009
Estimated GFR (ml/min/1.73 m^2)	72 ± 16	55 ± 23	0.0021
hs-CRP (mg/dl)	0.07 (0.04–0.10)	0.20 (0.10–0.61)	0.0053
BNP (pg/ml)	29 (13–56)	344 (74–1004)	<0.0001
Troponin T (ng/ml)	—	0.09 ± 0.34	—
HSP 60 (ng/ml)	7.32 (5.11–11.24)	12.34 (7.68–18.96)	0.0046
Cardiac catheterization			
Left ventricular end-diastolic pressure (mm Hg)	6.7 ± 3.5	11 ± 5	0.0002
Cardiac index (L/min/m^2)	2.9 ± 0.6	2.5 ± 0.6	0.0039
Left ventricular ejection fraction (%)	69 ± 9	42 ± 19	<0.0001
Echocardiography			
Left ventricular dimension at end-diastole (mm)	47 ± 5	57 ± 10	<0.0001
Left ventricular ejection fraction (%)	68 ± 9	40 ± 17	<0.0001
Cardiac deaths	—	11 (10%)	—
Rehospitalizations	—	26 (23%)	—
Angiotensin-converting enzyme inhibitors and/or angiotensin II receptor blockers	22 (35%)	83 (74%)	0.0003
β blockers	5 (8%)	50 (45%)	<0.0001
Calcium channel blockers	34 (55%)	18 (16%)	<0.0001
Spironolactone	1 (2%)	34 (30%)	<0.0001
Loop diuretics	2 (3%)	72 (64%)	<0.0001
Digoxin	3 (5%)	27 (24%)	0.0013
Statins	7 (11%)	18 (16%)	0.6036

Data are expressed as mean ± SD, number (percentage), or median (interquartile range).
hs-CRP = high-sensitivity C-reactive protein.

study, and the protocol was approved by the human investigations committee of our institution.

Blood samples were obtained at admission from all patients. The glomerular filtration rate (GFR) was estimated using the equation from the abbreviated Modification of Diet in Renal Disease (MDRD) study.[16] Because serum creatinine was measured using an enzymatic method, we used calibrated serum creatinine levels obtained by the following formula to estimate GFR: serum creatinine (Yaffe method) = 0.194 + 1.079 × serum creatinine (enzymatic method).[16] Transthoracic echocardiography was performed by experienced echocardiographers without knowledge of the biochemical data using an ultrasound instrument (Hewlett-Packard Sonos 7500; Philips Medical Systems, Andover, Massachusetts) equipped with a sector transducer (carrier frequency 2.5 or 3.75 MHz) <1 week after admission. Demographics and clinical data, including age, gender, and New York Heart Association (NYHA) functional class at admission, were collected from hospital medical records and patient interviews. Physicians were kept blind to the results of the biochemical markers, and optimal medical therapy that had been instituted was performed independently on the basis of measurements such as improvement

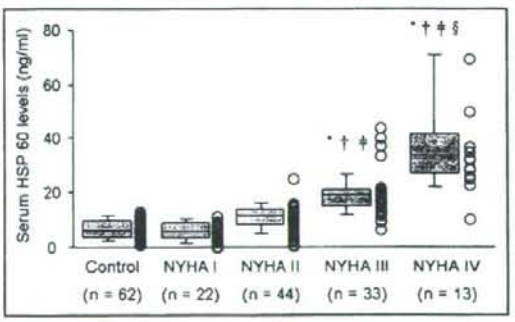

Figure 1. Association between serum HSP 60 level and NYHA functional class. *p <0.001 vs controls; †p <0.01 vs NYHA functional class I; ‡p <0.01 vs NYHA functional class II, and §p <0.01 vs NYHA functional class III.

in symptoms, physical examination findings, and pulmonary congestion on chest x-ray.[17] The diagnosis of hypertension, diabetes, and hyperlipidemia were obtained from medical records or patient histories of currently or previously received medical therapy. Serum HSP 60 levels were assessed

Table 2
Comparisons of clinical characteristics of 112 patients with chronic heart failure between those with and without cardiac events

Variable	Event Free (n = 75)	Cardiac Events (n = 37)	p Value
Age (yrs)	66 ± 13	67 ± 13	0.9636
Men/women	49/26	25/12	0.8139
NYHA functional class (I/II/III/IV)	17/39/15/4	5/5/18/9	<0.0001
Hypertension	39 (52%)	19 (51%)	0.9485
Diabetes mellitus	19 (25%)	11 (30%)	0.6231
Hyperlipidemia	18 (24%)	11 (30%)	0.5180
Current smokers	23 (31%)	6 (16%)	0.1074
Dilated cardiomyopathy	43 (57%)	21 (57%)	
Ischemic heart disease	32 (43%)	16 (43%)	0.9538
Blood examination			
Sodium (mEq/L)	142 ± 2.8	139 ± 4.1	0.0450
Creatinine (mg/dl)	0.83 ± 0.55	1.10 ± 0.48	0.0135
Estimated GFR (ml/min/1.73 m^2)	65 ± 23	50 ± 23	0.0077
hs-CRP (mg/dl)	0.29 (0.07–0.70)	0.31 (0.08–0.78)	0.2203
BNP (pg/ml)	305 (67–675)	741 (181–1530)	0.0059
Troponin T (ng/ml)	0.02 ± 0.04	0.29 ± 0.60	0.0078
HSP 60 (ng/ml)	11.13 (6.89–14.20)	18.18 (12.96–30.79)	0.0007
Cardiac catheterization			
Left ventricular end-diastolic pressure (mm Hg)	11 ± 6.3	13 ± 4.3	0.1667
Cardiac index (L/min/m^2)	2.6 ± 0.6	2.2 ± 0.4	0.0642
Left ventricular ejection fraction (%)	43 ± 19	39 ± 20	0.5451
Echocardiography			
Left ventricular dimension at end-diastole (mm)	56 ± 8	57 ± 13	0.7651
Left ventricular ejection fraction (%)	41 ± 17	39 ± 16	0.8099

Data are expressed as mean ± SD, number (percentage), or median (interquartile range).
Abbreviation as in Table 1.

using a colorimetric enzyme-linked immunosorbent assay test (HSP 60 ELISA Kit; StressGen Bioreagents Corporation, Ann Arbor, Michigan). The assay does not cross react with 100 ng/ml of other HSPs. The HSP 60 ELISA Kit has been certified for the detection of human HSP 60. The intra- and interassay coefficients of variation of the HSP 60 ELISA Kit have been determined to be <10%.

No patients were lost to follow-up (mean 569 ± 476 days, range 17 to 1,986) after admission to Yamagata University Hospital. Patients were prospectively followed up until the occurrence of cardiac events in every case. The end points were (1) cardiac death, defined as death from worsening CHF or sudden cardiac death, and (2) worsening CHF requiring readmission. Sudden cardiac death was defined as death without definite premonitory symptoms or signs and was established by the attending physician. A review of medical records and follow-up telephone interviews to survey cardiac events were conducted by senior cardiologists, who were blinded to blood examination data. Cardiac events were adjudicated using electrocardiography, chest x-rays, autopsy reports, death certificates, and witness statements.

Results are presented as mean ± SD for continuous variables and as percentages of the total number of patients for categorical variables. Skewed variables are presented as medians and interquartile ranges. Student's unpaired *t* test and the chi-square test were used for comparisons of continuous and categorical variables between 2 groups, respectively. If data were not distributed normally, the Mann-Whitney U test was used. Comparisons of data among NYHA functional classes and quartiles based on HSP 60 levels were performed using the Kruskal-Wallis test. A Cox proportional-hazards regression analysis was performed to evaluate the associations between cardiac events and measurements. Only variables with p values <0.05 on univariate Cox regression analysis were entered into multivariate Cox regression analysis. The cardiac event–free curve was computed according to the Kaplan-Meier method and compared using the log-rank test. All p values reported are 2 sided, and a p value <0.05 was considered significant. Statistical analysis was performed with a standard statistical program package (StatView version 5.0; SAS Institute Inc., Cary, North Carolina).

Results

The baseline clinical characteristics of patients with CHF and control subjects are listed in Table 1. Creatinine, high-sensitivity C-reactive protein, brain natriuretic peptide (BNP), HSP 60, left ventricular end-diastolic pressure, and left ventricular dimension at end-diastole were significantly higher in patients with CHF than in control subjects. The estimated GFR, cardiac index, and left ventricular ejection fraction were significantly lower in patients with CHF than in control subjects. As shown in Figure 1, serum HSP 60 levels were higher in patients with CHF than in control subjects and increased with advancing NYHA functional class, especially in patients with severe CHF in NYHA functional class IV.

There were 7 noncardiac deaths (3 from cerebral infarctions, 2 from gastric cancer, 1 from pneumonia, and 1 from renal failure) and 37 cardiac events (33%), including 11 cardiac deaths (3 in-hospital deaths) and 26 readmissions

Table 3
Univariate and multivariate analyses of predicting cardiac events in patients with chronic heart failure

Variable	Hazard Ratio	95% Confidence Interval	p Value
Univariate analysis			
Age*	1.138	0.800–1.644	0.4633
Gender (women vs men)	1.252	0.628–2.499	0.5230
NYHA functional class	1.857	1.292–2.671	0.0008
Presence of hypertension	1.232	0.646–2.349	0.5268
Diabetes mellitus	0.918	0.453–1.859	0.8115
Hyperlipidemia	0.816	0.403–1.652	0.5716
Sodium*	0.735	0.532–0.954	0.0406
Creatinine*	1.298	1.050–1.538	0.0338
Estimated GFR*	0.658	0.025–0.915	0.0102
hs-CRP*	1.249	0.964–1.621	0.0915
BNP*	1.448	1.001–2.463	0.0031
Troponin T*	1.121	0.961–1.134	0.1141
HSP 60*	1.373	1.147–1.884	0.0082
Stepwise multivariate analysis			
BNP*	1.361	1.002–2.063	0.0026
HSP 60*	1.268	1.046–1.441	0.0099
Estimated GFR*	0.721	0.545–0.954	0.0219
BNP*	1.409	1.002–2.064	0.0024
HSP 60*	1.301	1.047–1.544	0.0072
Sodium*	0.751	0.523–1.092	0.1354
Estimated GFR*	0.750	0.569–0.986	0.0411
BNP*	1.298	1.001–2.064	0.0375
HSP 60*	1.221	1.071–1.375	0.0046

* Per 1-SD increase.
Abbreviation as in Table 1.

for worsening heart failure during the follow-up period. The causes of cardiac death were worsening CHF in 9 patients and sudden cardiac death in 2 patients.

As listed in Table 2, patients with cardiac events were in higher NYHA functional classes compared with those without cardiac events. Furthermore, patients with cardiac events showed hyponatremia, renal dysfunction, and higher levels of BNP, troponin T, and HSP 60 compared with those without cardiac events. In contrast, other parameters, including age, gender, the numbers of patients with hypertension and diabetes mellitus, and cardiac catheterization and echocardiographic data, were not different between patients with and without cardiac events.

To determine risk factors to predict cardiac events, we performed univariate and multivariate Cox proportional-hazards regression analyses (Table 3). In the univariate analysis, HSP 60 was significantly associated with cardiac events (per 1-SD increase, hazard ratio 1.373, 95% confidence interval 1.147 to 1.884, $p = 0.0082$). Furthermore, NYHA functional class, sodium, creatinine, estimated GFR, and BNP were related to cardiac events significantly. Then, variables with p values <0.05 were entered into a stepwise multivariate Cox proportional-hazards regression analysis. Because creatinine and estimated GFR are similar parameters to reflect renal function, only estimated GFR was entered into the stepwise multivariate regression analysis. Estimated GFR, BNP, and HSP 60 were independent predictors of cardiac events among these variables on multivariate analysis.

Next, we classified all patients with CHF into 4 groups according to serum HSP 60 levels: ≤7.67 ng/ml (n = 28, first quartile), 7.68 to 13.06 ng/ml (n = 28, second quartile), 13.07 to 18.96 ng/ml (n = 28, third quartile), and ≥18.97 ng/ml (n = 28, fourth quartile). The patients in the fourth quartile were older and were in higher NYHA functional classes compared with those in the lower 3 quartiles. Furthermore, the patients in the fourth quartile showed hyponatremia, renal dysfunction, and higher levels of high-sensitivity C-reactive protein, BNP, troponin T, and HSP 60 compared with those in the lower 3 quartiles. In contrast, other parameters, including gender, the numbers of patients with hypertension and diabetes mellitus, and cardiac catheterization and echocardiographic data, were not significantly different among quartiles of HSP 60 levels. As shown in Figure 2, the Cox proportional-hazards regression analysis revealed that the fourth quartile was associated with the highest risk for cardiac events among quartiles of HSP 60 levels (7.638-fold compared with the first quartile, $p < 0.01$). Furthermore, Kaplan-Meier analysis demonstrated that the fourth quartile had a significantly higher cardiac event rate compared with the lower 3 quartiles ($p < 0.0001$; Figure 2).

Discussion

In the present study, we showed the following new and important findings: (1) serum HSP 60 was significantly elevated in patients with CHF compared with healthy control subjects; (2) HSP 60 levels increased with advancing NYHA functional class; (3) HSP 60 levels were higher in patients with cardiac events compared with those without cardiac events; (4) multivariate Cox proportional-hazard regression analysis demonstrated that HSP 60, BNP, and estimated GFR were independent factors to predict adverse clinical outcomes in patients with CHF; and (5) the fourth quartile of HSP 60 level was associated with the highest risk for cardiac events compared with the lower 3 quartiles.

Previous studies have demonstrated that the overexpression of HSP 60 prevents apoptosis by ischemia-reoxygenation in myocardial cells,[6,7] and the overexpression of HSP in transgenic mice protected the heart against damaging effects of ischemia.[8] Thus, it is conceivable that HSP 60 represents a protective function in patients with chronic stress, as in CHF. HSP 60 was previously considered to be located only intracellularly. However, recent reports have shown that HSP 60 is present in the circulation of healthy human subjects, and circulating HSP 60 levels are increased significantly associated with cardiovascular diseases.[11,18,19] The release of HSP 60 might be part of a response to protect neighboring cells against various stresses, and increased circulating HSP 60 levels might reflect underlying cell stress levels. Although the detailed mechanism and source of HSP 60 release in CHF has been unknown to date, HSP 60 release could be from the cells of tissues experiencing chronic stresses. Because CHF is accompanied by a variety of pathologic changes that trigger disease progression, a multiaxis framework was proposed to more completely appreciate the pathophysiology of CHF. BNP, troponin T, and creatinine assess different pathophysiologic mechanisms in CHF; BNP is secreted from the ventricles by mechanical

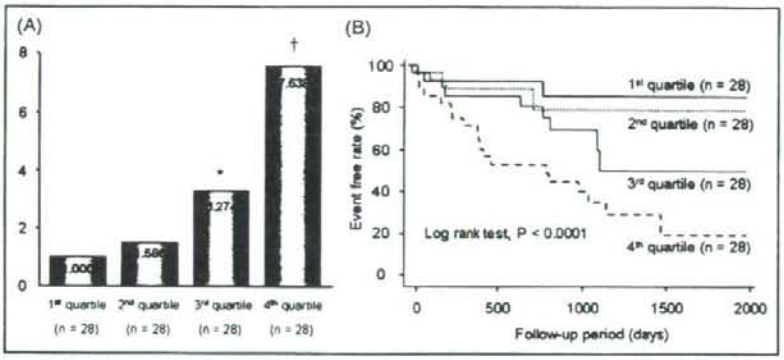

Figure 2. Patients were divided into 4 groups on the basis of HSP 60 levels: ≤7.34 ng/ml (n = 50, first quartile), 7.35 to 12.85 ng/ml (n = 50, second quartile), 13.86 to 18.68 ng/ml (n = 50, third quartile), and ≥18.69 ng/ml (n = 49, fourth quartile). *(A)* Hazard ratios relative to first quartile. *p <0.05 and †p <0.01 vs first quartile. *(B)* Kaplan-Meier analysis of cardiac event-free rate among the 4 groups.

overload, troponin T is a marker for ongoing myocardial cell injury, and creatinine is a marker of renal function. Therefore, the combined measurement of HSP 60 (a marker for various stress levels in CHF) and other markers (BNP, troponin T, creatinine, etc.) might be a highly reliable evaluation for risk-stratifying patients with CHF. Because several studies have reported that cytosolic proteins leak from cardiomyocytes in patients with CHF, suggesting the presence of ongoing myocardial damage by necrosis, apoptosis, and so on,[20,21] HSP 60 might be a novel marker of myocardial damage in patients with CHF. We will compare HSP 60 with troponin T and heart-type fatty acid–binding protein with regard to the detection of ongoing myocardial damage in patients with CHF in a future study. It is necessary to find a detailed mechanism of HSP 60 release and the functional roles of circulating HSP 60.

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Repeated Waon therapy improves pulmonary hypertension during exercise in patients with severe chronic obstructive pulmonary disease

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Abstract
Objectives: Repeated Waon therapy, which uses a far infrared-ray dry sauna system, improved the vascular endothelial function and the cardiac function in patients with chronic heart failure. In patients with chronic obstructive pulmonary disease (COPD), pulmonary hypertension (PH) is associated with a poor prognosis. We investigated whether repeated Waon therapy improves PH, cardiac function, exercise tolerance, and the quality of life (QOL) in patients with COPD.
Methods: Consecutive 13 patients with COPD, who met the Global Initiative for Chronic Obstructive Lung Disease criteria and had breathlessness despite receiving conventional treatments, were recruited for this study. They underwent Waon therapy at 60 C in sauna for 15 min following 30 min warmth with blankets outside of the sauna room. This therapy was performed once a day, for 4 weeks. Cardiac function, exercise tolerance, and St. George's Respiratory Questionnaire (SGRQ) were assessed before and 4 weeks after Waon therapy.
Results: Right ventricular positive dP/dt at rest elevated significantly from 397 ± 266 to 512 ± 320 mmHg/s ($p = 0.024$) after the therapy. While the PH at rest did not significantly decrease, the PH during exercise decreased significantly from 64 ± 18 to 51 ± 13 mmHg ($p = 0.028$) after Waon therapy. Furthermore, the therapy prolonged the mean exercise time of the constant load of cycle ergometer exercise test from 360 ± 107 to 392 ± 97 s ($p = 0.032$). The total scores of SGRQ improved from 59.7 ± 16.9 to 55.3 ± 17.2 ($p = 0.002$). In addition, no adverse effects were observed related to Waon therapy.

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Conclusions: Repeated Waon therapy improved right ventricular positive dP/dt, PH during exercise, exercise tolerance and the QOL in patients with severe COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic, debilitating and fatal lung disease. Many patients with COPD suffering from breathing-related problems in spite of receiving conventional therapies such as medication, physical therapy and oxygen inhalation [1]. Therefore, there is a great demand for the development of new therapies for COPD.

In patients with COPD, pulmonary hypertension (PH) is associated with a poor prognosis. Remodeling of the pulmonary vessels is the principal causative factor of PH in COPD. PH is a common complication of COPD, and its presence is associated with shorter survival and worse clinical course [2]. However, severe PH at rest is uncommon in patients with COPD [3]. Kessler et al. assessed the evolution of pulmonary hemodynamics in a group of 131 patients with moderate COPD who did not have PH at rest, although 76 patients (58%) developed PH during exercise [4].

We developed a form of thermal therapy, namely Waon therapy (soothing warm therapy) that differs from the traditional sauna [5]. We previously reported that repeated Waon therapy improved the vascular endothelial function in persons with risk factors for atherosclerosis [6]. Furthermore, this therapy improved the cardiac function and exercise tolerance in patients with congestive heart failure, who suffered from chronic symptoms despite the administration of full medications, and it was also performed safely in patients with high risk [7,8]. These effects are attributable to the reduction in the cardiac preload and afterload [7]. We also have reported that the mechanism of improvement of vascular function due to an overexpression of endothelial nitric oxide synthase (eNOS) in Syrian golden hamsters [9]. We consider that the eNOS upregulation induced by sauna is caused by increases in the cardiac output and blood flow, which in turn results in an increased shear stress. Therefore, we expect that repeated Waon therapy may relieve the symptoms in COPD patients by improving PH.

The present study investigated whether repeated Waon therapy improves PH, cardiac function, exercise tolerance, and the quality of life (QOL) in patients with severe COPD.

Subjects and methods

Study group

We studied consecutive 13 patients with COPD who satisfied the criteria of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guideline (i.e. $FEV_1/FVC < 70\%$ after bronchodilator agent administration). They had symptoms such as cough and dyspnea with grade 4 or 5 of Medical Research Council (MRC) dyspnea scale in spite of conventional treatments, no acute exacerbation of COPD in the past 3 months and no signs of any respiratory tract infection. All patients have already received medications for COPD, such as inhaled β_2-agonists, anticholinergics and oral theophyllines. The medications and pulmonary rehabilitation programs had not been changed for at least 4 weeks before and during this study. Written informed consent was obtained from all of the patients before participation. This protocol was approved by the Ethics Committee of the Faculty of Medicine, Kagoshima University.

Waon therapy

Waon therapy used a far infrared-ray dry sauna which is evenly maintained at 60 °C and differs from traditional sauna. Waon therapy has an absence of hydration pressure, and was performed as previously reported [7]. Briefly, the patients were placed in a supine position on a bed in a 60 °C sauna for 15 min, and then after leaving the sauna, they were then underwent bed rest with a blanket to keep them warm for an additional 30 min. All patients were weighed before and after the therapy, thereafter oral hydration with water was used to compensate for the lost weight.

Waon therapy was performed once a day, 5 days a week for 4 weeks, a total of 20 times. To rule out acute effects of Waon therapy, all examinations were performed before the first treatment and on the next day after the last treatment.

Measurements

Physical examinations

The blood pressure (BP), pulse rate, body weight and body temperature were measured before and after Waon therapy.

Cardiac function tests

Echocardiography was performed to evaluate the cardiac function and to determine the tricuspid regurgitation speeds by the continuous wave Doppler echocardiography, in which the pulmonary systolic pressure was estimated by the use of Bernoulli's equation. The LV and RV functions were analyzed by the Tei index, the sum of isovolumetric contraction time and isovolumetric relaxation time divided by ejection time [10]. Right ventricular (RV) positive dP/dt at rest was assessed by using tricuspid regurgitation (TR) estimated with Doppler echocardiography before and after Waon therapy. Two points at 1.0 and 2.0 m/s were selected on the rising segment of the TR velocity curve and the time interval (Δt) between them was measured. The increase in instantaneous velocity between the two points was converted to RV-right atrial pressure gradient (ΔP) using the simplified Bernoulli equation. The rate of RV pressure rise was obtained as $\Delta P/\Delta t$ [11].

In addition, we performed echocardiograms during ergometer exercise to monitor the changes in the pulmonary arterial pressure. Studied patients pedaled a bicycle (Aerobic Exercise Ergometer, STB-1200; Nihon Kohden; Tokyo, Japan) with a 10 W load for 3 min and after that, the load of the bicycle increased by 10 W for additional every 3 min. And they kept pedaling at the speed of 60 rpm and were encouraged to continue exercising for as long as possible. We recorded TR velocity by the continuous wave Doppler echocardiogram every 3 min.

We evaluated exercise tolerance by ergometer. At first, the patients pedaled a bicycle without a load for 3 min and after that, the load of a bicycle increased by 10 W every minute. They pedaled a bicycle at the speed of 60 rpm. The patients were encouraged to continue exercising for as long as possible. The test was terminated at symptom limitation or if there were any safety concerns. We monitored oxygen saturation by pulse oximeter (SpO_2), BP, heart rate (HR) and ECG during exercise.

St. George's Respiratory Questionnaire (SGRQ) scores

The health-related quality of life was evaluated with the Japanese versions of St. George's Respiratory Questionnaire (SGRQ) which is a measure of impaired health in disease of chronic airflow limitation. SGRQ contains 50 items that can be divided into three dimensions (symptoms, activity and impacts), and their scores ranged from 0 to 100 (worst status) [12].

Laboratory measurements

A blood sample was obtained to measure the plasma levels of the brain natriuretic peptide (BNP) with a radioimmunoassay, hematocrit and albumin before and after repeated Waon therapy.

Statistical analysis

All data are expressed as the mean value ± S.D. The data before and after Waon therapy were compared using the paired Student's t-test. A p-value of <0.05 was considered to be significant.

Table 1 Patient's profile

Case	Age	Gender	Height (cm)	BW (kg)	BMI (kg/m^2)	%FEVI	GOLD stage	MRC scale	SI (pak-year)
1	67	M	161.5	56.0	20.5	18.9	IV	5	88
2	73	M	155.0	37.4	15.6	34.3	IV	5	68
3	80	M	160.9	70.3	27.1	34.2	IV	5	50
4	69	M	156.0	47.0	19.3	34.4	III	4	34
5	67	M	166.3	61.5	22.2	11.9	IV	5	60
6	76	M	166.1	43.4	15.7	40.2	IV	5	92
7	76	M	163.7	43.4	16.1	37.5	IV	5	46
8	86	M	161.8	54.5	20.8	48.5	III	5	45
9	77	M	159.8	46.0	18.0	37.5	III	5	61
10	79	M	150.6	51.1	22.7	51.5	II	4	60
11	71	M	163.0	49.0	18.4	39.7	IV	4	45
12	75	M	164.0	56.2	20.9	61.2	IV	4	80
13	78	M	155.4	57.2	23.7	57.5	II	4	114

M, male; BW, body weight; BMI, body mass index; GOLD, Global Initiative for Chronic Obstructive Lung Disease; MRC, Medical Research Council; SI, smoking index.

Table 2 Physical findings and laboratory examinations

	Before therapy	After therapy	*p*-Value
BW (kg)	50.8 ± 7.8	50.9 ± 7.8	0.926
HR (bpm)	86 ± 9	85 ± 13	0.820
SBP (mmHg)	130 ± 17	120 ± 15	0.002
DBP (mmHg)	74 ± 9	69 ± 8	0.0002
Hematocrit (%)	40.3 ± 9.2	40.0 ± 8.5	0.617
Alb (g/dl)	4.1 ± 0.3	4.1 ± 0.3	1.0
BNP (pg/ml)	25.7 ± 19.6	21.3 ± 13.4	0.294

Value given as mean ± S.D.; BW, body weight; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; Alb, albumin; BNP, brain natriuretic peptide.

Results

Clinical characteristics

The clinical characteristics of 13 patients are summarized in Table 1. All patients were male with a mean age of 74.9 ± 5.5 years (range, 67–86 years). The mean BMI was 20.1 ± 3.4, and the mean $\%FEV_1$ was 39.0 ± 13.8%. They were all past smokers. According to the GOLD classification, eight patients were in stage IV (very severe), while 3 patients were in stage III (severe), and the other 2 patients were in stage II (moderate). Eight patients were MRC dyspnea scale grade 5 and 5 patients were grade 4.

Effects of Waon therapy on body weight, heart rate, blood pressure and laboratory variables

We performed repeated Waon therapy without any problems for all enrolled patients. None of the patients experienced dyspnea in the sauna room. Physical findings and laboratory examinations before and after repeated Waon therapy are demonstrated in Table 2. All patients did not have any cardiac disorders either during or after Waon therapy. The mean body weight and the mean HR did not substantially change before and after Waon therapy. On the other hand, systolic and diastolic blood pressure decreased significantly (systolic blood pressure: 130 ± 17 to 120 ± 15, $p = 0.002$; diastolic blood pressure: 74 ± 9 to 69 ± 8, $p = 0.0002$) after Waon therapy. The hematocrit, albumin and plasma BNP concentrations did not change. The MRC dyspnea scale grades did not change. In addition, the liver function, renal function and electrolytes did not change after Waon therapy (data not shown).

Cardiac function

Cardiac function was evaluated by the use of echocardiography before and after repeated Waon therapy. The ejection fraction and systolic pulmonary artery (PA) pressure at rest did not change after the therapy in comparison to baseline. The RV Tei index tended to decrease after the therapy (0.51 ± 0.15 to 0.46 ± 0.11, Table 3). RV positive dP/dt elevated significantly from 397 ± 266 to 512 ± 320 mmHg/s ($p = 0.024$) after the therapy (Table 3). No patients had any symptoms or signs of heart failure during this therapy.

Exercise tolerance

The PA pressure at rest did not improved after repeated Waon therapy. However, the PA pressure during exercise decreased significantly after

Table 3 Echocardiographic parameters

	Before therapy	After therapy	*p*-Value
LVDd (mm)	41.0 ± 5.7	39.2 ± 5.6	0.184
LAD (mm)	30.3 ± 7.2	28.8 ± 8.7	0.923
EF (%)	68.8 ± 8.8	67.8 ± 8.8	0.602
LV Tei index	0.41 ± 0.08	0.43 ± 0.13	0.534
RV Tei index	0.51 ± 0.15	0.46 ± 0.11	0.169
Systolic PAP (mmHg)	41.0 ± 8.3	37.4 ± 6.4	0.168
IVC (mm)	9.2 ± 1.5	9.3 ± 1.6	0.675
RV dP/dt (mmHg/s)	397 ± 266	512 ± 320	0.024

Value given as mean ± S.D.; EF, ejection fraction; PAP, pulmonary artery pressure.

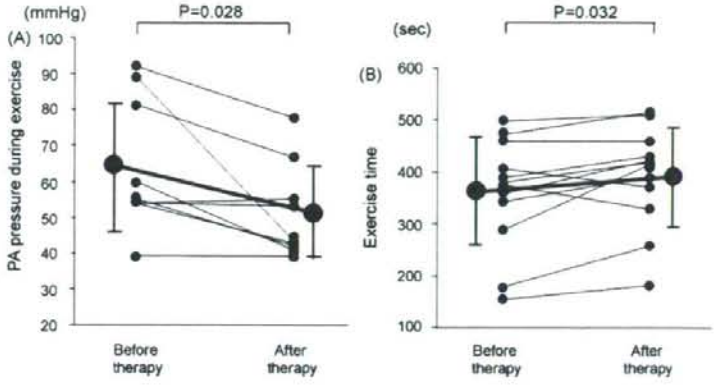

Figure 1 PA pressure during exercise (A) and exercise time (B) before and after 4 weeks of Waon Therapy. The mean of pulmonary arterial pressure during exercise decreased significantly after Waon therapy with the same loading dose of exercise. The exercise time significantly prolonged after Waon therapy. PA = pulmonary artery.

repeated Waon therapy in comparison to baseline (64.0 ± 18.0 to 51.3 ± 13.1 mmHg, $p = 0.028$), with the same loading dose of exercise (Fig. 1A). The exercise time also significantly increased after repeated Waon therapy (359.6 ± 106.5 to 391.5 ± 97.0 s, $p = 0.032$) (Fig. 1B). In addition, the lowest SpO_2 during exercise significantly elevated after the therapy (89.4 ± 4.8 to 91.3 ± 4.1%, $p = 0.022$) (Fig. 2).

St. George's Respiratory Questionnaire (SGRQ) scores measurements

SGRQ scores were measured to evaluate the change of QOL, which is consisted of 50 questionnaires about symptoms, activity and impacts, before and after the repeated Waopn therapy. The symptoms scores significantly decreased after repeated Waon therapy in comparison to baseline (67.7 ± 20.6 to 56.5 ± 23.5, $p = 0.007$). In addition, the total scores and the impacts scores in SGRQ significantly decreased (59.7 ± 16.9 to 55.3 ± 17.2, $p = 0.002$, 43.7 ± 21.8 to 40.1 ± 21.2, $p = 0.024$). The activity scores tend to decrease (83.3 ± 16.4 to 81.2 ± 15.0) (Fig. 3).

In contrast, the MRC did not change because it is a questionnaire only about dyspnea.

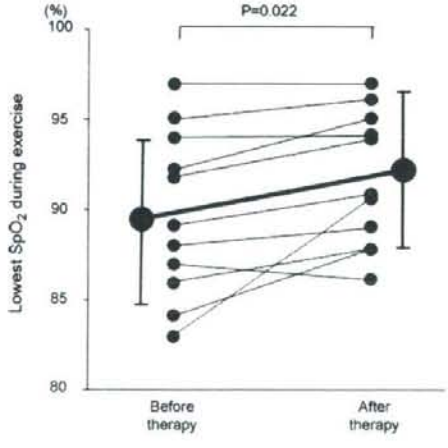

Figure 2 Changes of SpO_2 during exercise before and 4 weeks after Waon Therapy. SpO_2 during exercise is increased significantly.

Discussion

This is the first report to demonstrate that Waon therapy regimen improves PH during exercise, exercise time, in addition, the symptoms, impacts and total scores of SGRQ in the patients with severe COPD.

We suppose that the prolongation of exercise time by Waon therapy is related to the pulmonary arterial pressure during exercise, not at rest. An improvement of ventilation competence may be related to the prevention of desaturation during exercise. Previous studies have identified a number of mechanisms for exercise-induced PH in COPD, including hypoxic vasoconstriction, reduction of the capillary bed by emphysema, extramural compression by increased alveolar pressure or impaired release of endothelium-derived relaxing factors. These factors may together contribute to the development of PH during exercise, and dynamic hyperinflation due to the expiratory flow limitation that results in increased alveolar pressure [13–16].

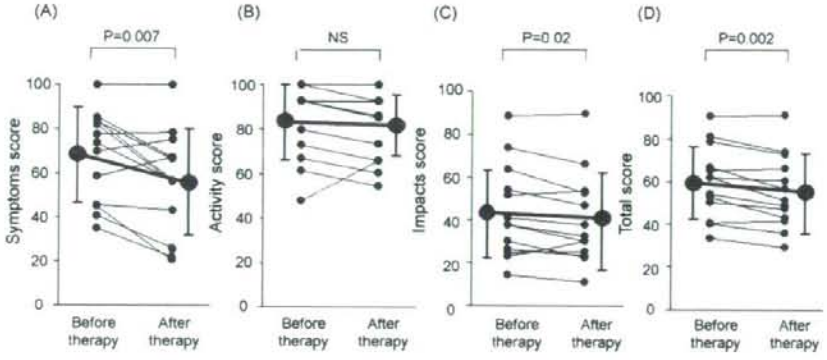

Figure 3 Changes of SGRQ symptoms score (A), activity score (B), impacts score (C), total score (D) before and 4 weeks after Waon Therapy. The symptoms, the impacts and the total scores significantly decreased after repeated Waon therapy. SGRQ = St. George's Respiratory Questionnaire.

Kubo et al. examined the relationship between vessel remodeling and the physiology of pulmonary circulation in severe COPD patients who underwent lung-volume-reduction surgery [17]. They analyzed the pulmonary hemodynamics at rest and during exercise, and the morphology of the pulmonary arteries in these patients. As a result, they have described that pulmonary artery remodeling leads to a reduced recruitability and distensibility of the pulmonary vessels and it is therefore closely related to exercise PH. Therefore, they suggested that the degree of the remodeling cannot be estimated by the resting pulmonary artery pressure. The expression of eNOS in pulmonary arteries is reduced in COPD patients with PH [18] and also in smokers [19]. The diminished synthesis of nitric oxide may contribute to the alterations in the structure and endothelial function of pulmonary vessels in cigarette-smoke-induced respiratory disease. In addition, the structural and functional changes of pulmonary circulation are apparent at the initial stages of COPD [19]. Recent investigations have shown endothelial dysfunction and the changes in the expression of endothelium-derived mediators that regulate vascular tone and cell growth in the pulmonary arteries of patients with mild COPD. The expression of eNOS decreases in the pulmonary arteries of COPD patients with pulmonary hypertension [20].

We previously demonstrated that the gene expression and protein level of eNOS increase significantly in the peripheral arteries from the golden hamster after 4-week repeated Waon therapy [9]. Moreover, we also reported this therapy improves the impaired vascular endothelial function in a setting of coronary risk factors [6]. We suggest that this therapy improves endothelial function in COPD patients in the same way. The significant decrease in BP after this therapy is probably due to an improved endothelium-dependent vasodilation. Anconina et al. described RV dP/dt provides a reliable noninvasive index to approach RV contractility [11]. According to our data, RV dP/dt elevated significantly after the therapy, suggesting the improvement of RV contractility. We expect that Waon therapy increase the expression of eNOS in PA, and improve PA vascular function. Thereafter, RV contractility improved by reducing the afterload of RV. As a result, we therefore assume that the PH during exercise and the exercise tolerance also improved.

The present study indicates that 4-week Waon therapy regimen was found to improve symptoms and QOL in patients with COPD. Oga et al. reported the SGRQ total score to be a predictive factor of mortality, independent of FEV_1 and age [21]. In addition, Domingo-Salvany et al. reported the SGRQ total score to be independently associated with the total and respiratory mortality in Cox models, including age, FEV_1, and BMI [22]. It was also notable that the SGRQ total score improved by Waon therapy in 4 weeks. Improvements in the symptoms score contributed to most of the improvements in the total score. We previously reported that a self-assessment QOL of the patients with chronic heart failure improved after this therapy [8]. In the items of a concrete questionnaire regarding symptoms, the frequency of a cough, respiratory symptomatic frequency and the period of being well conditioned were improved in this study. Ernst et al. suggested that regular sauna bathing may reduce the incidence of acute respiratory infections [23]. No patient demonstrated any respiratory tract infection during this study.

Masuda et al. reported that repeated Waon therapy may be useful for mildly depressed patients with appetite loss and other subjective complaints [24]. Patients with severe COPD are at an increased risk of developing depression [25]. We therefore suppose that this therapy may diminish the psychological distress in patients with COPD. Further studies are needed to investigate the change in the SGRQ scores and the frequency of acute exacerbations by continuing Waon therapy for a longer time period.

Study limitations

This study is not a case–control study and the study group was also very small. As a result, it remains necessary to investigate more COPD patients, including those with mild COPD. We also need to evaluate the airway inflammation in COPD after this therapy.

In addition, we need a further evaluation of the vascular endothelial function and changes in the pulmonary arterial pressure in COPD by continuing such Waon therapy for a longer period of time.

Conclusion

The repeated Waon therapy was thus found to improve the RV positive dP/dt, PH during exercise, exercise tolerance and SGRQ scores in the patients with severe COPD. This therapy may therefore be a novel, safe, and promising therapy for patients with severe COPD.

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