Myocardial Expression Level of Neural Cell Adhesion Molecule Correlates with Reduced Left Ventricular Function in Human Cardiomyopathy

Nagao et al: NCAM Is Up-regulated in Human Failing Heart

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Journal Subject Codes: Heart failure: [10], Diagnostic testing: [33], Myocardial biology: [155]
Abstract

Background—We recently screened for cardiac genes induced by metabolic stress and identified neural cell adhesion molecule (NCAM) as a candidate. This study aimed to clarify the expression pattern of neural cell adhesion molecule (NCAM) in human cardiomyopathy.

Methods and Results—Sixty-four cardiac tissue samples of dilated cardiomyopathy (DCM) patients were dichotomized according to the immunohistochemically determined signal intensity of NCAM staining (NCAM-high and NCAM-low groups). Clinical and hemodynamic data of the patients were compared between the two groups. Fibrosis area, left ventricular (LV) end-diastolic volume index and LV diastolic pressure were greater in the NCAM-high group (22.8% vs. 11.6%, P<0.05; 130.3 ± 57.6 ml/m² vs. 104.8 ± 31.7 ml/m², P<0.05; 14.3 ± 8.0 mmHg vs. 8.8 ± 4.7 mmHg, P<0.005; respectively). Incidence of cardiac death and admission for worsening heart failure were higher in the NCAM-high group over a follow-up of 6.3 years (log rank P<0.05). Another 18 tissues were analyzed to determine the relationships between expression level of NCAM and major metabolic genes as well as hemodynamic parameters. The mRNA level of NCAM correlated with the serum (r=0.58, P=0.01) and mRNA levels (r=0.61, P=0.008) of brain-derived natriuretic peptides. It was also correlated with the mRNA levels of proliferator-activated receptor-γ coactivator-1 α (r=0.69, P=0.002), and nuclear respiratory factor 1 (r=0.74, P<0.001).

Conclusions—Expression of NCAM was associated with worsening hemodynamic parameters and major metabolic genes. Together with our previous findings, these data support the involvement of NCAM in LV remodeling, revealing new insights into the pathophysiology of heart failure.

Key Words: neural cell adhesion molecule, heart failure, cardiomyopathy
Heart failure (HF) is characterized by diverse molecular, cellular, and physiological changes in the myocardium, resulting in adverse left ventricular (LV) remodeling. Among various pathophysiological changes in failing hearts, altered energetics in cardiac myocytes has been known to be closely linked to LV remodeling. Indeed, myocardial ATP levels in advanced HF are reduced by approximately 30%. As a result, cardiac myocytes suffer from metabolic stress, which plays an important role in the progression of irreversible cardiac fibrosis and LV remodeling. Thus, identifying new molecules regulated under conditions of impaired energy metabolism may potentially further the understanding of HF.

For this purpose, we previously set-up a screening method to search for genes up-regulated under conditions of decreased cellular ATP levels in cardiomyocytes. In the screening process, we specifically targeted cell surface proteins because they are easily accessible for exogenous drugs or have potential as novel biomarkers. One of the genes we identified was neural cell adhesion molecule (NCAM). NCAM belongs to the immunoglobulin superfamily of cell adhesion molecules. It has been elucidated that NCAM is highly expressed in neural tissues where not only by mediating intercellular adhesions, but by inducing downstream signaling, NCAM plays a major role in cell survival, development, migration and neurite outgrowth.

In the previous analysis, we found that: (1) NCAM was induced by treatment with oligomycin, a mitochondrial F0-F1 ATP synthase inhibitor, in cardiac myocytes; (2)
endogenous NCAM or stimulation of NCAM-mediated signaling by treatment with NCAM-derived peptides played a protective role after oligomycin treatment in cardiac myocytes; (3) NCAM was remarkably up-regulated by more than 24-fold during the LV remodeling period in a rat model of hypertension-induced HF; (4) its expression was unique in its heterogeneous pattern; up-regulation of NCAM started in the subendocardium area where expression of NCAM was closely associated with fibrotic change and then expanded throughout the myocardium during the advance of LV remodeling. These results indicate that NCAM may be involved in LV remodeling and have potential as a new therapeutic target or a biomarker in the clinical setting. Therefore, confirming the expression of NCAM in human failing hearts is of great significance.

Reports about the expression pattern of NCAM in human failing hearts are scarce and it is currently unknown whether NCAM is also up-regulated in the human failing heart as we observed in the animal model. Accordingly, we sought to investigate the expression pattern of NCAM in human LV tissue. We analyzed the relationships between NCAM expression levels and hemodynamic parameters, as well as the expression levels of major genes involved in metabolic regulation.
Methods

Research design and patient population

We analyzed the expression of NCAM in the tissues of LV endomyocardial biopsies (EMBs) and examined the relationships between its level and hemodynamic parameters as well as other gene expression levels in DCM patients. EMB was performed at Osaka Red Cross Hospital to determine the etiology of HF, especially to rule out myocarditis and other specific myocardial diseases, according to the following institutional criteria; 1) new onset HF with clinical symptoms of dyspnea, chest pain, or palpitation, 2) unexplained impairment of LV function and/or LV dilatation, 3) no evidence of coronary artery disease or primary valvular disease. These criteria were in agreement with a later published guideline on EMBs. Final diagnoses was obtained on the basis of clinical history, laboratory examinations, electrocardiogram, echocardiography, disease-specific tests such as computed tomography scan and nuclear imaging, biopsies from extra-cardiac tissues, as well as histopathological analysis of EMB tissue. This study is consisted of following two groups of patients.

Immunohistochemical analysis group

Between March 2000 and July 2007, 64 patients were diagnosed with dilated cardiomyopathy (DCM) after undergoing EMB. We retrospectively examined EMB tissues obtained from these patients for immunohistochemical analysis. All patients underwent left heart catheterization and 59 (92%) patients also underwent right heart catheterization. All patients
provided written informed consent for the procedure and subsequent analyses.

Gene expression analysis group

We examined 18 EMB tissues obtained from DCM patients who underwent EMB between February 2011 and October 2012 to analyze the relationships between gene expression level of NCAM and hemodynamic parameters. All patients underwent left heart catheterization and 17 patients also underwent right heart catheterization. For 1 patient, we did not perform left ventricular angiography because of the reduced renal function. All patients provided written informed consent for the procedure and gene expression analyses. The ethics committee of Osaka Red Cross Hospital approved the study protocol.

Heart catheterization and endomyocardial biopsy

In right heart catheterization, pulmonary artery pressure, pulmonary artery wedge pressure, right ventricular pressure, right atrial pressure and cardiac index (C.I.) were obtained using a triple-lumen Swan-Ganz catheter. In left heart catheterization, selective coronary angiography was performed to rule out coronary artery disease after aortic pressure recording. Thereafter, LV pressure recordings and left ventricular angiography was performed using a pigtail catheter to measure the LV end-diastolic pressure, LV systolic pressure, LV end-diastolic volume index and LV end-systolic volume index. Finally, LV EMB was performed via the femoral artery using myocardial biopsy forceps (Technowood, Tokyo, Japan) after retrograde passage of the aortic valve. Tissues were obtained from the LV endomyocardium in each
patient. No procedural complications occurred in the study population.

**Immunohistochemistry**

Immediately after being obtained, biopsy tissues were fixed in 10% buffered formalin and embedded in paraffin. Serial sections were obtained and stained with hematoxylin and eosin and Masson’s trichrome stain. When required, disease-specific analyses, such as Congo red or Gomori-trichrome staining, was also performed. The remaining paraffin blocks were stored for further immunohistochemistry. For histological examination of NCAM, we obtained additional sections that were sequential with sections stained with Masson’s trichrome to evaluate the relationship between the expression pattern of NCAM and fibrotic change. Immunohistochemical staining of NCAM was performed using a conventional ABC method as described previously. To eliminate interparative variability in the immunohistochemical data, 10 sections were immunostained in a single batch using identical aliquots of diluted antibodies and other reagents.

**Histopathological analysis**

For each specimen, we selected the largest or most well-preserved tissue for histopathological analysis. Images of each section were acquired using a microscope at a magnification of x40, which covered almost the whole sample area. The percentage area of fibrosis was calculated by dividing the sum of the fibrotic areas of the section by that of the total tissue area as described previously. For semi-quantitative analysis of NCAM staining, three areas cut in a
plane parallel to the long axis of the cardiac myocytes were selected at a magnification of x200 in each section, where staining of NCAM in intercalated discs was most clearly observed. The signal intensity of NCAM staining was analyzed in 10 randomly selected intercalated discs in each area using Image J software, and the average signal intensity of NCAM was calculated as NCAM intensity value in each specimen. Accordingly, we analyzed NCAM expression at 30 points per specimen. The details of the method of quantification are described in the results. When the intensity value of NCAM staining exceeded the median value (68.4 arbitrary units), the specimen was categorized into the NCAM-high group, whereas those categorized into the NCAM-low group had values below the median value. The analysis was performed in a blinded manner.

**Follow up**

Clinical follow up was performed retrospectively in the patients included in the immunohistochemical analysis until March 2012 for a median duration of 6.3 years (IQR 4.2-9.3 years) after EMB was performed. Clinical information was obtained either from a review of the medical records, by telephone or by letter contact with the patients or family members. Clinical follow up information for the NCAM-high and the NCAM-low groups was obtained from 30 patients (93.8%) and 29 patients (90.6%) patients at 1 year, 28 patients (87.5%) and 28 patients (87.5%) at 3 years, and 16 patients (50.0%) and 23 patients (71.9%) at 6 years.
RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated and purified using TRIzol reagent (Invitrogen, California, USA), and cDNA was synthesized from 1 μg of total RNA using a Transcripter First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) in accordance with the manufacturer’s instructions. For RT-PCR, the reaction was performed using a SYBR Green PCR master mix (Applied Biosystems, California, USA), and the products were analyzed using a thermal cycler (ABI Prism 7900HT sequence detection system). The levels of β-actin transcripts were used to normalize cDNA levels.

Gene-specific primers were as follows:

NCAM sense, 5’ GTGTGTTACAGCGGAGGAT-3’
antisense, 5’ GATGACATCTGGCCTTTGT-3’

PGC-1α sense, 5’ TGACACAACACGGACAGAAC-3’
antisense, 5’ GCATCACAGGTATAACGGTAGG-3’

NRF1 sense, 5’ GGCACTGTCTCACTTATCCAGGTT-3’
antisense, 5’ CAGCCACGGCAGAATAATTCA-3’

Tfam sense, 5’ AATGGATAGGCAGGAAACC-3’
antisense, 5’ CAAGTATTATGCTGGCAGAAGTC-3’

β-actin sense, 5’ AGGCACCAGGGCGTGAT -3’
antisense, 5’ TCGTCCCAGTTGGTGACGAT -3’
Statistics

Frequency analysis was performed by $\chi^2$ test. Continuous variables were expressed as the mean value ± SD or median with interquartile range. The differences in continuous variables between the two groups were assessed by Student t test or by nonparametric Mann Whitney U test as appropriate. Survival rates were calculated using the Kaplan-Meier method and were compared using the log-rank test. Correlations were tested with the use of the Spearman correlation coefficient or Pearson correlation coefficient as appropriate. Probability values <0.05 were regarded as statistically significant.

Results

Histopathological findings of biopsy specimens

To analyze the expression pattern of NCAM in human remodeling hearts, and especially its relationship with fibrosis, we performed immunostaining of NCAM and Masson’s trichrome staining in sequential sections of specimen from DCM patients. In tissues where fibrotic change was scarce and normal-appearing cardiac myocytes remained intact, staining of NCAM was very weak and restricted to the intercalated discs (Figure 1A). However, in sections where massive fibrotic change occurred, strong staining of NCAM was observed in intercalated discs and sarcolemma in cardiac myocytes adjacent to the fibrotic area (Figure 1B).
Semi-quantitative analysis for expression level of NCAM

A total of 64 DCM patients were included in the immunohistochemical analysis. The mean ejection fraction (EF) was 40±13% and the mean LV end-diastolic volume index (LVEDVI) was 118±48 ml/m² (Table 1). We performed semi-quantitative analysis of the expression level of NCAM based on the digitally determined strength of NCAM immunoreactivity as described in the Methods and Figure 2. We categorized the 64 patients into two groups according to the median value of NCAM signal intensity; an NCAM-low group (intensity value < 68.4 arbitrary units (a.u.)) and an NCAM-high group (intensity value ≥ 68.4 a.u.). There was no difference in clinical background or medications between the NCAM-low and NCAM-high groups (Table 2).

Comparison of fibrosis and hemodynamic parameters between the two groups

The extent of the histologically determined fibrotic area was significantly larger in the NCAM-high group compared with the NCAM-low group (22.8% (IQR: 10.9-38.7%) vs. 11.6% (IQR: 6.3-17.1%), P<0.05, Table 3). LVEDVI and LV diastolic pressure (LVDP) of patients categorized into the NCAM-high group were also significantly higher than those in the NCAM-low group (130.3±57.6 ml/m² vs. 104.8±31.7 ml/m², P<0.05; 14.3±8.0 mmHg vs. 8.8±4.7 mmHg, P<0.005; respectively). No difference was observed in EF, mean aortic pressure (mAoP), mean pulmonary artery pressure (mPAP), mean pulmonary capillary wedge pressure (mPCWP), or C.I. between the two groups (Table 3).
Clinical follow-up outcomes

During the follow up period, there were 3 cardiac deaths, 5 non-cardiac deaths and 9 rehospitalizations for worsening HF. The cumulative incidence of all-cause death was not different between NCAM-low and NCAM-high groups (29.8% vs. 6.9%, log-rank P=0.98) (Figure 3). The cumulative incidence of adverse outcome defined by the combined endpoint of cardiac death and admission for worsening HF were significantly higher in the NCAM-high group compared with NCAM-low group at 1 year (19.2% vs. 0%, log-rank P=0.01), at 3 years (22.4% vs. 3.7%, log-rank P=0.03), at 6 years, (30.7% vs. 7.6%, log-rank P=0.02), and in the overall cohort (31.5% vs. 19.1%, log-rank P=0.03).

Relationships between NCAM gene expression and hemodynamic parameters

To further clarify the relationship between the expression level of NCAM and LV remodeling, we examined mRNA from LV EMB tissues. qRT-PCR analysis revealed that the cardiac expression level of NCAM was significantly correlated with serum level and cardiac expression level of brain natriuretic peptides (BNP) (r=0.61, P=0.008, r=0.58, P=0.001, respectively), (Figure 4A and 4B). A modest correlation was observed between cardiac expression level of NCAM and PCWP (r=0.44, P=0.07) (Figure 4C). No correlation was observed between the expression level of the NCAM gene and LVEF, LVEDVI and C.I. (Figure 4D, 4E and 4F).
Relationship between expression of NCAM and metabolic genes

To elucidate whether up-regulation of NCAM in human LV tissues was associated with response to metabolic stress, we analyzed the relationship between the expression of NCAM and genes involved in cardiac energy metabolisms. As shown in Figure 5A and 5B, the expression of NCAM was significantly correlated with those of PPAR\(_\gamma\)-coactivator-1 \(\alpha\) (PGC-1\(\alpha\)), a major regulator of mitochondrial function and respiration in the heart \((r=0.69, P<0.005)\), and its downstream genes, nuclear respiratory factor 1 (NRF1) \((r=0.74, P<0.0005)\). No significant correlation was observed between the expression level of NCAM and transcription and mitochondrial maintenance factor (Tfam) (Figure 5C).

Discussion

In our previous report, we performed functional screening in combination with a signal sequence trap method and identified that NCAM was up-regulated in cardiac myocytes under conditions of inhibition of mitochondrial ATP production. The mRNA levels of NCAM were also found to be remarkably up-regulated by 24-fold during the remodeling period of hypertrophy to HF in a Dahl salt-sensitive rat model.\(^{14}\)

In the present study, we reported that: (1) NCAM was weakly expressed in the intercalated discs in normal-appearing human LV tissues, and it was strongly expressed in the myocardium adjacent to the areas suffering from massive fibrotic change, which were consistent with our previous observations in a rat model of hypertension-induced HF\(^ {14}\); (2)
more advanced LV remodeling was observed in the NCAM-high group as indicated by more severe fibrosis, larger LVEDVI and higher LVDP compared with the NCAM-low group; (3) in accordance with these pathological and hemodynamic results, higher incidences of adverse cardiac events were observed in the NCAM-high group compared with the NCAM-low group; (4) up-regulation of NCAM expression in cardiac tissue was associated with higher hemodynamic overload as indicated by increased level of circulating or expressing BNP; (5) NCAM mRNA levels were correlated with the expression levels of metabolic genes such as PGC1 alpha and NRF1. These results support our previous work suggesting the involvement of NCAM-mediated signaling in the pathophysiology of LV remodeling.

Reports regarding the expression pattern of NCAM in the heart are scarce. Gattenlohner et al screened for genes differentially expressed in human ischemic cardiomyopathy and identified NCAM as a candidate molecule. In their report, they demonstrated that NCAM was strongly expressed in cardiomyocytes adjacent to fibrotic areas in rat and human cardiac tissues, which was similar to our observation in DCM tissues. Therefore, a common up-stream pathway for inducing expression of NCAM may exist in the perifibrosis area in ischemic cardiomyopathy and DCM. Arnett et al recently performed a genome-wide association study of echocardiographic phenotypes in hypertensive individuals and identified NCAM as a genetic predictor of LV wall thickness and LV mass. In their report, they demonstrated that NCAM may be directly connected with numerous genes that

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have been shown to be involved with cardiac phenotypes. Taken together, expression of NCAM may be closely related to LV function and remodeling in a variety of human cardiac diseases.

Whether up-regulation of NCAM in human LV tissue was triggered by metabolic stress consistent with our initial screening concept is of great interest. Because the energy demands in cardiac myocytes are quite high and must be precisely regulated, mechanisms to maintain energy homeostasis are known to exist. PGC-1α has been shown to be a major regulator of mitochondrial function, biogenesis and respiration in many tissues including the heart. 34-36 Rohas et al demonstrated that under conditions of metabolic stress leading to decreased cellular ATP level, PGC-1alpha levels were induced remarkably and enhanced mitochondrial gene expression. 37 This system worked as a compensatory system for maintaining cellular ATP level and cell survival. 38, 39 In this context, increased expression of PGC-1alpha may indicate the existence of metabolic remodeling. Thus, we tried to determine the relationship between the expression levels of PGC-1alpha and NCAM. The results demonstrated that the expression level of NCAM was closely related with that of PGC-1alpha and its downstream target NRF1, suggested that induction of NCAM expression might be associated with a compensatory response of PGC-1alpha during metabolic stress. Unfortunately, directly evaluating metabolic status, such as myocardial ATP content or the activity of metabolic
enzymes was limited by their instability and the small amounts of tissue available from LVEMB.

Ditlevsen et al showed that stimulation of NCAM-mediated signaling with a synthetic NCAM-ligand peptide protected neuronal cells against apoptosis, thereby showing the protective function of NCAM.40 Likewise, results in our previous experiments using NCAM-siRNA or pharmacological tools for NCAM stimulation demonstrated that NCAM-mediated signaling played a protective role under conditions of inhibition of mitochondrial ATP production in cardiac myocytes.14 Therefore, it would be reasonable to postulate that up-regulation of NCAM expression in the human remodeling heart could be a compensatory response against cell death. In this regard, augmentation of NCAM-mediated signaling by recently developing NCAM-mimetic peptides may have therapeutic potential for cardiac diseases.41-43 Future analysis using genetically manipulated animal models may clarify the function and mechanism of expression of NCAM in vivo in the failing heart.

Limitations

There are several limitations to the present study. First, the present study on the expression of NCAM in human LV tissues was based on the analysis of biopsy specimens, which represent regional molecular changes in the failing heart. Thus, the results of relationships between the molecular changes in sections and whole heart hemodynamics may need careful interpretation. Second, we did not analyze biopsy specimens of normal human control hearts.
However, variable degrees of LV dysfunction in the patients included in this study provided the opportunity to demonstrate the relationship between fibrosis, hemodynamic parameters and NCAM expression. Third, regarding the relationships between the expression of NCAM and metabolic genes, we only analyzed the correlation of mRNA levels by qRT-PCR; therefore, we cannot establish the causality. Finally, our evaluation was limited by relatively small number of patients. In the cohort analysis, the low event rate precluded multivariate analysis. However, findings in human tissue demonstrated here reinforce and expand our previous observations in cells and animal models.

In conclusion, we showed for the first time that expression of NCAM was associated with worsening hemodynamic parameters and the expression of major metabolic genes in human failing hearts, as observed in the animal model. NCAM may be involved in LV remodeling, which provides new insights into pathophysiology of HF.

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energy pathways, mediated by hub metabolites’ (3307), from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to K.O.).

Disclosures

None.

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Continuous variables are shown as mean ± SD, and other values are n (%).

ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker.
Table 2. Baseline clinical characteristics of patients with DCM

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<td>59 ±13</td>
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Amiodarone 2 (6) 0 (0) 0.15

Continuous variables are shown as mean ± SD, and other values are n (%). ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker.
Table 3. Comparison of fibrosis area and hemodynamic parameters between the NCAM-low and NCAM-high groups

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</tr>
<tr>
<td>EF (%)</td>
<td>40.0±11.2</td>
<td>39.7±14.6</td>
<td>0.95</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>8.5 (6.0-12.0)</td>
<td>13.0 (7.3-19.0)</td>
<td>0.001</td>
</tr>
<tr>
<td>PCWP (mmHg)</td>
<td>8.0 (5.0-11.0)</td>
<td>9.0 (6.0-17.0)</td>
<td>0.17</td>
</tr>
<tr>
<td>C.I. (L/min/m²)</td>
<td>2.6 (2.2-3.4)</td>
<td>2.8 (2.2-3.3)</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Continuous variables are shown as mean ± SD or median (interquartile).

C.I., cardiac index; EF, ejection fraction; LVEDP, left ventricular end-diastolic pressure; LVEDVI, left ventricular end-diastolic volume index; PCWP, pulmonary capillary wedge pressure.
Figure Legends

Figure 1

(A and B); Images of tissues with mild (A) and severe (B) fibrotic change obtained from DCM patients. In each group, two representative images are shown. Left; Masson’s trichrome staining. Middle; NCAM staining, Right; high power field of the square area shown in the middle panels. Left and middle panels are sequential sections.

Figure 2

Semi-quantitative analysis of NCAM staining.

In each tissue, three areas were selected where intercalated discs were most clearly observed. 1280x1024 pixel size photomicrographs were captured (A, left) and transferred into eight-bit black and white pictures (A, right). In general, the strongest intensity points (white) were restricted to the immune-reactive material recognized by anti-NCAM antibodies at the intercalated discs. Thus, 10 intercalated discs were selected at random for analysis of the signal intensity of NCAM in each area. Signal intensity was calculated by deleting minimum (background) from maximum signal intensity obtained by using Image J software (B). The average of NCAM signal intensity in 30 points (10 intercalated discs in each of three areas) was calculated as the NCAM intensity value in each tissue. (C); Histogram of NCAM signal intensity values in 64 specimens. When the intensity value of NCAM staining achieved the
cut-off level (68.4 arbitrary units; median), the tissue was categorized into the NCAM-high group, and samples were categorized into the NCAM-low group when the value was below the cut-off level.

Figure 3
Mortality and cardiac death/admission for worsening HF
Kaplan-Meier survival for (A) all cause mortality and (B) cardiac death and admission for worsening HF

Figure 4
Correlation between NCAM gene expression and hemodynamic parameters.
BNP, brain natriuretic peptide; C.I., cardiac index; EF, ejection fraction; EDVI, end-diastolic volume index; PCWP, pulmonary capillary wedge pressure

Figure 5
Correlation between the expression level of NCAM and metabolic genes
PGC1α, peroxisome proliferator-activated receptor gamma coactivator -1 alpha; NRF1, nuclear respiratory factor 1; Tfam, mitochondrial transcription factor A
Myocardial Expression Level of Neural Cell Adhesion Molecule Correlates with Reduced Left Ventricular Function in Human Cardiomyopathy

Kazuya Nagao, Naoya Sowa, Katusmi Inoue, Motoko Tokunaga, Kohei Fukuchi, Koji Uchiyama, Haruyasu Ito, Fujio Hayashi, Toshinori Makita, Tsukasa Inada, Masaru Tanaka, Takeshi Kimura and Koh Ono

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