Original Article

Gene Polymorphism of Myospryn (Cardiomyopathy-Associated 5) Is Associated with Left Ventricular Wall Thickness in Patients with Hypertension

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We examined a gene polymorphism of a novel Z-disc–related protein, myospryn (cardiomyopathy-associated 5). We focused on one haplotype block associated with a tag single nucleotide polymorphism (SNP) that covered 16 of 27 coding SNPs with linkage disequilibrium (minor allele frequency 0.413). Screening a myospryn polymorphism (K2906N) in a general health check-up of a rural Japanese population revealed an association with cardiac diseases (p=0.0082). In further analysis of the interaction between K2906N and cardiac function in patients, K2906N was associated with the anteroseptal wall thickness of the left ventricle in a recessive model (p=0.0324) and with the ratio of the peak velocity of the early diastolic filling wave to the peak velocity of atrial filling (A/E) (p=0.0278). In an association study based on left ventricular wall thickness, we found a significant difference in the K2906N genotype between controls and patients with cardiac hypertrophy. These results suggest that the K2906N polymorphism could be clinically associated with left ventricular hypertrophy and diastolic dysfunction independent of known parameters. Although the precise mechanism underlying this association remains to be elucidated, treatment with angiotensin II induced an increase in heart myospryn mRNA level *in vitro* and *in vivo*. Our results suggest that the polymorphism of myospryn is associated with left ventricular hypertrophy, and an association between a Z-disc protein and cardiac adaptation in response to pressure overload. (*Hypertens Res* 2007; 30: 1239–1246)

***Key Words*: diastolic dysfunction, genetics, cytoskeleton**

Introduction

Cardiac hypertrophy usually follows an increase in workload imposed on the heart. Although mechanical, neural, and endocrine mechanisms are important in this process, individual myocardial responses to workload are variable. For instance, there is a poor correlation between blood pressure and left ventricular (LV) hypertrophy in individual study participants (*1*). It has been reported that the LV mass of endur-

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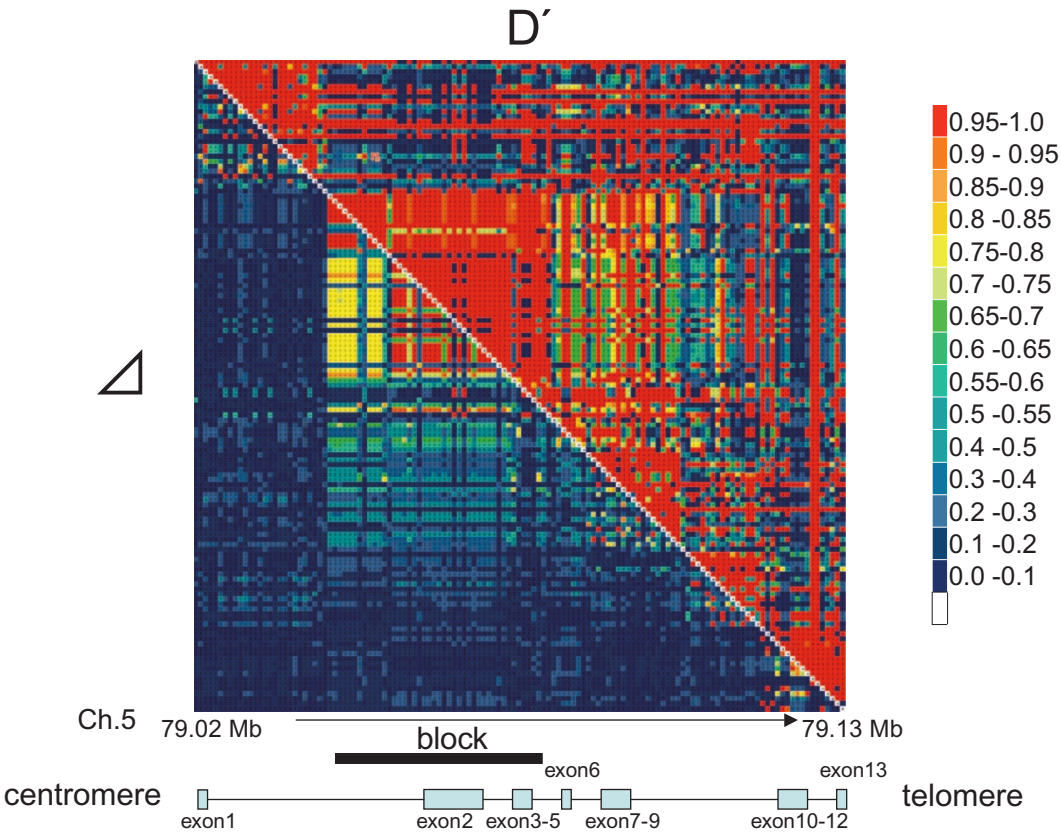

Fig. 1. *Linkage disequilibrium (LD) blocks and genomic structures in myospryn (chromosome 5 [Ch.5]; position 79.02 Mb–79.13 Mb). Pairwise LD indices of D´ and Δ are presented in the upper right and lower left of the rectangle, respectively. The color gradient indicates the value of LD. LD blocks (top), locations of the haplotype block (middle), and exons (bottom) are shown.*

ance athletes with similar training habits increases to different degrees, and that the amount of training accounts for only 11% of this variability (*2*).

Mechanotransduction, the conversion of a mechanical stimulus into a cellular response, fills important functions in the myocardium, where each cycle of contraction and relaxation leads to dynamic deformations (*3*). Stretching induces muscle growth in mechanical overload and hypertension (HT)-induced hypertrophy (*4*), and the renin-angiotensin system is involved in this process. Recently, it was shown that the angiotensin II type 1 receptor itself can receive a stretch signal though a ligand-independent mechanism (*5*). Thus, polymorphisms of renin-angiotensin system–related genes are associated with the development of cardiac hypertrophy in patients (*6*, *7*). Although a variety of cellular transduction elements, including humoral factors, channels, and second messengers (*8*), mediate the response to mechanical stretch, recent studies suggest that the cytoskeletal structure and related Z-disc proteins may also play a role (*9*, *10*). Indeed, the physiologic importance of these elements is supported by the fact that mutation of these proteins is associated with human dilated cardiomyopathy (*11–15*). Recent studies have reported that the novel Z-disc–related protein myospryn is expressed in striated muscle cells, where it co-localizes with sarcomeric α-actinin (*16*, *17*). In this study, we further examined the relationship between myospryn polymorphisms and alterations of cardiac function in patients with HT.

Methods

General Populations of the Towns of Tanno and Sobetsu

The subjects were 1,132 inhabitants (435 men and 697 women) of two towns (Tanno and Sobetsu) in a rural area of Hokkaido, the northernmost island of Japan, who had undergone health examinations in 2002 (*18*). The ages of the subjects ranged from 40 to 92 years, 19.2% of the subjects being over the age of 65. Since the two towns are in a rural area, most of the subjects were engaged in agriculture. Blood samples were collected from all of the subjects after overnight fasting, and systolic (SBP) and diastolic blood pressure (DBP) was measured at rest in a sitting position. Fasting plasma glucose level (FPG) and plasma levels of total choles-

Table 1. Frequency of 27 SNPs in Coding Region of Human Myospryn Gene by Sequencing DNA Samples from 50 Japanese Volunteers and Comparison with Hapmap Information

Allele	NCBI reference	Tag SNP	r^2/test	Minor allele frequency
191A/G (Y64C)	rs16877109	rs6859595	1	0.413*
1046G/A (G349D)	rs1366271	rs6859595	1	0.413*
1772G/A (G591D)	rs16877124	rs6859595	1	0.413*
3017T/C (V1006A)	rs6893869	rs6859595	1	0.413*
3925A/G (I1309V)	rs16877133		1	0.413*
3998C/T (A1333V)	rs16877135	rs6859595	1	0.413*
4700C/A (A1567E)	rs1428223		1	0.413*
4795T/G (S1599A)	rs1428224	rs6859595	1	0.413*
5138T/A (N1713I)	rs16877141	rs6859595	1	0.413*
5161A/G (I1721V)	rs1428225		1	0.413*
5624C/T (V1875A)	rs16877147	rs6859595	1	0.413*
5750A/G (D1917G)	rs16877150	rs6859595	1	0.413*
5758A/G (S1920G)	rs16877151		1	0.413*
6784G/C (L2262V)	rs6859595	rs6859595 (tag SNP)		0.413*
8718A/C (K2906N)	rs2278239	rs6859595	1	0.413*
10074C/G (H3358Q)	rs3828611	rs6859595	1	0.413*
3884C/T (V1295A)	rs4704585	rs4704585 (tag SNP)		0.352
569A/G (D190G)	rs10942901			0.173
8803G/A (G2935A)	rs2278240			0.088
11780G/A (R3927Q)	rs1129770			0.088
10747A/G (K3583E)	rs12514461			0.074
4138A/G (I1380V)	rs13158477			0.035
5006T/C (L1669S)	rs1019762			0.035
525G/T (Q175H)	rs6895605			0
5174C/T (L1725S)	rs17254174			0
7147A/G (K2383E)	rs7721884			0
12188C/T (P4063L)	rs10043986			0

SNP, single nucleotide polymorphism; NCBI, National Center for Biotechnology Information. *Indicates linkage disequilibrium.

terol (TC), neutral lipids (TG), and high-density lipoprotein cholesterol (HDL) were determined by standard methods. We calculated low-density lipoprotein cholesterol (LDL) by the Friedwald formula, and non–high-density lipoprotein cholesterol (non-HDL) by subtracting HDL from TC. Data on medical history and current condition were obtained from the subjects in interviews with staff members of the health clinic in each town. Subjects with FPG above 126 mg/dL and/or who were currently being treated for diabetes were considered to have diabetes mellitus (DM). Subjects with SBP above 140 mmHg and DBP above 90 mmHg and those taking antihypertensive agents were considered to have HT. Subjects with TC above 220 mg/dL, TG above 150 mg/dL, or HDL below 40 mg/dL were considered to have hyperlipidemia (HL). Informed consent was obtained from all patients, and all procedures were carried out in accordance with institutional and national ethical guidelines for human studies. The study protocol was approved by the ethics committees of Sapporo Medical University and Osaka University Graduate School of Medicine.

Patients and Ultrasound-Echocardiographic Examination

A total of 174 patients were enrolled, most of whom were diagnosed with high blood pressure (defined as SBP >140 mmHg and/or DBP >90 mmHg on repeated measurements, or as current treatment with medication for HT). Blood samples were obtained in the morning after an overnight fast. Informed consent was obtained from all patients, and all procedures were carried out in accordance with institutional and national ethical guidelines for human studies. The study protocol was approved by Ethical Review Committee for Human Genome Research of Osaka University Graduate School of Medicine.

Comprehensive two-dimensional echocardiography was performed using a cardiac ultrasound unit (Sonos 5500; Hewlett-Packard, Palo Alto, USA). Measurements included interventricular septal thickness (IVSTd), posterior wall thickness (PWTd), LV diameter at end-diastole (LVDd) and LV diameter at end-systole (LVDs). Fractional shortening was calculated as (LVDd – LVDs)/LVDd. LV mass was esti-

Table 2. Clinical Characteristics According to K2906N Allele Status in General Population

	AA	AC	CC	*p*
Subjects (*n*)	278	576	278	
Age (years)	64.2±0.7	64.5±0.5	64.2±0.7	n.s.
Sex (male: %)	44.6	36.6	36.0	0.004
Height (cm)	156.7±0.5	155.8±0.4	155.5±0.5	n.s.
Weight (kg)	58.9±0.6	57.6±0.4	57.8±0.6	n.s.
BMI (kg/m^2)	23.9±0.7	22.7±0.1	23.9±0.2	n.s.
SBP (mmHg)	138.8±1.4	137.0±1.0	139.3±1.5	n.s.
DBP (mmHg)	75.4±0.7	75.8±0.5	76.3±0.7	n.s.
PR (/min)	68.1±0.7	69.2±0.5	69.8±0.7	n.s.
Cardiac disease (%)	6.9±1.4	5.4±1.0	5.1±1.4	0.0082
Stroke (%)	1.8±0.7	1.2±0.5	1.4±0.5	n.s.
Hypertension (%)	30.3±2.8	31.1±1.9	31.4±2.8	n.s.
Diabetes (%)	4.7±1.4	6.4±1.0	5.8±1.4	n.s.
Hyperlipidemia (%)	7.9±1.6	6.9±1.1	8.3±1.6	n.s.
Adiponectin (mg/dL)	7.44±0.24	8.13±0.17	7.72±0.24	n.s.
Creatinine (mg/dL)	1.01±0.38	0.97±0.17	0.97±0.16	0.0496
BUN (mg/dL)	16.2±0.25	15.8±0.17	15.7±0.25	n.s.
UA (mg/dL)	5.08±0.07	4.94±0.05	5.02±0.07	n.s.
T-chol (mg/dL)	199±2	200±1	202±2	n.s.
TG (mg/dL)	107±4	105±3	104±4	n.s.
HDL (mg/dL)	55.9±0.8	55.2±0.5	55.5±0.8	n.s.
Na (mEq/L)	142.7±0.1	142.4±0.1	142.4±0.1	n.s.
K (mEq/L)	4.23±0.02	4.24±0.01	4.24±0.02	n.s.
Calcium (mg/dL)	9.39±0.02	9.40±0.01	9.42±0.02	n.s.
AST (IU/L)	25.1±0.6	24.4±0.4	24.1±0.58	n.s.
ALT (IU/L)	22.5±0.7	21.6±0.5	21.7±0.7	n.s.
FPG (mg/dL)	98.0±1.2	99.0±0.9	99.1±0.7	n.s.
HbA1c (%)	5.26±0.04	5.30±0.03	5.29±0.04	n.s.
IRI (μU/mL)	4.84±0.22	4.86±0.15	5.27±0.22	n.s.
CRP (mg/dL)	0.101±0.007	0.094±0.005	0.101±0.007	n.s.

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; PR, pulse rate; BUN, blood urea nitrogen; UA, uric acid; T-chol, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein cholesterol; AST, aspartate aminotransferase; ALT, alanin aminotransferase; FPG, fasting plasma glucose; IRI, insulin resistance index; CRP, C-reactive protein.

mated using the formula validated by Devereux and Reichek (*19*):

$$\text{LV mass (g)} = 1.04 \times [(\text{IVSTd} + \text{PWTd} + \text{LVDd})^3 - \text{LVDd}^3] - 13.6.$$

LV mass was normalized against body surface area and expressed as LV mass index. To assess LV diastolic function, the diastolic filling of LV (LV inflow) was examined using Doppler echocardiography. The LV diastolic filling pattern was obtained with the sample volume at the tips of the mitral valve in the apical four-chamber view and recorded at the end-expiratory phase during quiet breathing (*20*). The peak velocity of the early diastolic filling wave (E wave) and the peak velocity of atrial filling (A wave) were recorded, and the E-to-A ratio (A/E) was calculated.

Northern Blot Analysis

Equal aliquots of total RNA (15 μg) were separated by 1% formaldehyde-agarose gel electrophoresis, and hybridization and washing were performed as previously described (*21*). Loading conditions were determined by ethidium bromide staining of 18S and 28S ribosomal RNA. Northern analysis was also performed with multiple tissue Northern blots prepared from high-quality poly(A)+ RNA normalized for a β-actin hybridization signal (Clontech) according to the manufacturer's instructions. The myospryn (corresponding to nucleotides 10,852–12,210) and MLC-2v (full-length myosin light heavy chain-2v) cDNAs were labeled with [^{32}P]dCTP using the RadPrime DNA Labeling System (Invitrogen, Carlsbad, USA). Unincorporated nucleotides were removed by spin column chromatography (Sephadex G-50; Amersham Biosciences, Buckinghamshire, UK).

Table 3. Analysis of Serum Creatinine and Heart Disease in Recessive Model

	AA	AC+CC	*p*
Subjects (*n*)	278	854	
Creatinine (mg/dL)	1.0±0.01	0.97±0.01	0.01
Cardiac disease (%)	6.9	5.3	0.04

Table 4. Case Control Study of Left Ventricular Wall Thickness

	Case	Control	*p*
Subjects (*n*)	81	93	
Age (years)	63.6±1.3	60.5±1.2	0.09
Sex (male: %)	67.9	45.2	0.003*
BMI (kg/m^2)	23.7±0.4	23.0±0.3	0.2
Hypertension (%)	78.5	56.3	0.003*
SBP (mmHg)	166.8±3.4	154.3±3.3	0.009
DBP (mmHg)	96.4±2.0	90.7±1.9	0.04
IVSth (mm)	12.0±0.1	8.6±0.1	<0.0001*
PWth (mm)	11.6±0.2	9.3±0.2	<0.0001*
K2906N polymorphism (*n* (%))			0.006*
AA	27 (33.3)	12 (12.9)	
AC	32 (39.5)	48 (51.6)	
CC	22 (27.2)	33 (35.5)	
Allele (*n* (%))			0.009
A	86 (53.1)	72 (38.7)	
C	76 (46.9)	114 (61.3)	

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; IVSth, thickness of interventricular septum; PWth, posterior left ventricular wall thickness. *Indicates significant difference ($p<0.00625$) evaluated by *t*-test with Bonferroni adjustments.

Cardiac Myocyte Culture and Animal Models of Cardiac Hypertrophy

Cardiac myocytes were obtained from the ventricles of 1-day-old Sprague-Dawley rats, and were isolated and cultured as previously described (*21*). Using this method, >95% of the cells were identified as cardiac myocytes, as assessed by immunofluorescent staining with an anti-cardiac myosin heavy chain (MHC) antibody. Viability was assessed by cell number, frequency of contractions (*i.e.*, intrinsic heart rate), cellular morphology, and trypan blue exclusion. Angiotensin II (1 μmol/L) and phenylephrine (20 μmol/L) showed no observable effects on cellular viability at the concentrations used for all treatment conditions.

Male C57/BL6 mice (8 weeks old, 20–25 g) were used for experiments. After the mice were anesthetized with intraperitoneal ketamine (80 mg/kg) and xylazine (10 mg/kg), an osmotic minipump (Alzet model 2004; Alza, Mountain View, USA) containing either angiotensin II (infusion rate 100 or 500 ng/kg/min) or vehicle was implanted in the midscapular region for 4 weeks. All experiments were approved by the Osaka University Committee on Animals.

Statistical and Haplotype Analyses

Values are expressed as means±SEM. All statistical analyses were performed using the JMP 5.1 statistical software package (SAS Institute, Cary, USA) or StatView 5.0 software (SAS Institute). Data were compared using Student's unpaired *t*-test for comparisons between two groups and ANOVA followed by Dunnett's test for multiple comparisons with Bonferroni adjustments. Haplotype frequencies were estimated with the expectation-maximization (EM) algorithm, using the web-based program HapMap (http://www.hapmap.org/index.html.en). The linkage disequilibrium indices D' and Δ were calculated using spreadsheet software (Excel).

Results

Human Myospryn Polymorphisms

From the Hapmap database (http://www.hapmap.org/index.html.en), in the locus of human myospryn (chromosome 5; position 79.02 Mb–79.13 Mb) we focused on one haplotype block with a powerful tag single nuclear polymorphism (SNP) (rs6859595) in which 17 SNPs were in linkage disequilibrium, as shown in linkage disequilibrium blocks (Fig. 1). We also selected 27 SNPs in the coding region of the human myospryn gene from the Ensembl databases (http://www.ensembl.org/Homo_sapiens/geneview?gene=ENSG00000164309), as shown in Table 1; this haplotype block covered exons 2–5, and its tag SNP also covered 12 SNPs in linkage disequilibrium among 27 coding SNPs. We examined the frequency of each of the 27 SNPs by utilizing direct polymerase chain raction (PCR)—whose primers were designed in the exon or intron to amplify each exon including the SNPs—or TaqMan PCR. The SNP frequency of DNA samples from healthy volunteers as control subjects showed that 4 additional coding SNPs, located in exons 1 and 10–12, were in linkage disequilibrium. Importantly, a total of 16 SNPs showed linkage disequilibrium with significant frequency (minor allele frequency 0.413), which almost covered all exons of the myosoryn gene, as shown in Table 1. Thus, in subsequent experiments we focused on the K2906N myospryn polymorphism because it is a very powerful SNP.

Human Myospryn Polymorphism in a General Population

The present study enrolled 1,132 inhabitants of two rural Japanese towns. The subsequent experiments focused on analysis of the myospryn K2906N polymorphism (minor allele frequency 0.48). As shown in Table 2, analysis of K2906N

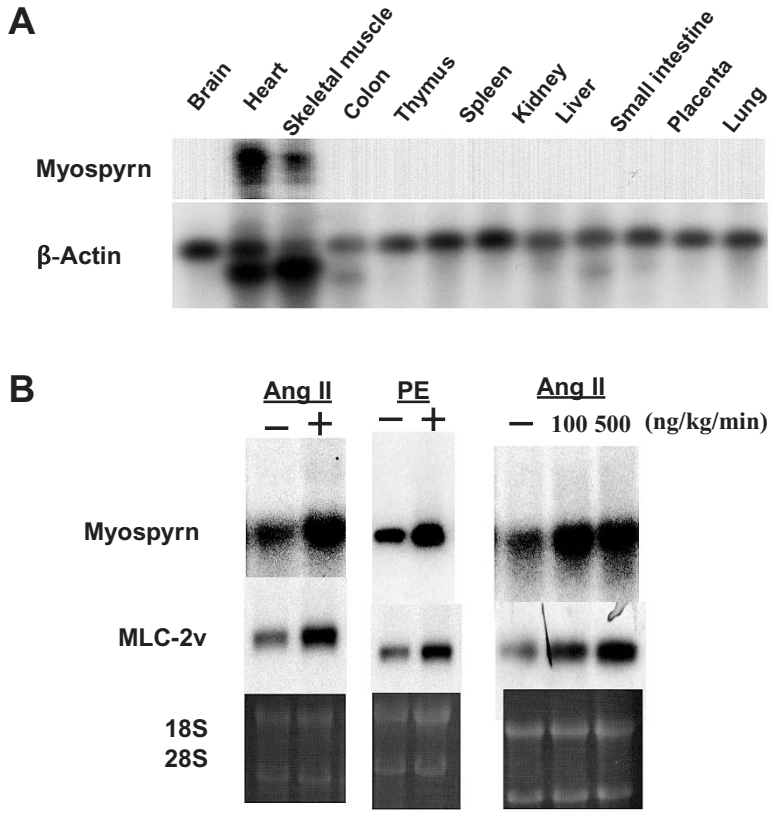

Fig. 2. *Analysis of myospryn expression. A: Northern blot of myospryn in several human tissues. B: Northern blot of myospryn and myosin light chain (MLC)-2v in rat neonatal cardiomyocytes (left and middle) and mouse heart (right). Loading conditions were determined by ethidium bromide staining of 18S and 28S ribosomal RNA. In the left and middle panels, Ang II indicates angiotensin II (1 μmol/L), and PE indicates phenylephrine (20 μmol/L). In the right panel, Ang II (angiotensin II: 100 or 500 ng/kg/min) indicates systemic infusion into C57 Bl/6 mice for 4 weeks.*

revealed an association with cardiac diseases in a general check-up (genotype AA: 6.9%, AC: 5.4%, CC: 5.1%, $p=0.0082$), and serum creatinine level (AA: 1.01 mg/dL, AC: 0.97 mg/dL, CC: 0.97 mg/dL, $p=0.0496$). In analysis using a recessive model, the K2906N polymorphism was associated with cardiac disease (AA: 6.9%, AC+CC: 5.3%, $p=0.04$) and serum creatinine (AA: 1.0 mg/dL, AC+CC: 0.97 mg/dL, $p=0.01$) (Table 3). Since cardiac diseases diagnosed through a general check-up could include heart failure, cardiac myopathy, and cardiac hypertrophy, we further examined the association between this polymorphism and cardiac function using ultrasound-echocardiography. Although serum creatinine level normally reflects renal damage, another marker, blood urea nitrogen (BUN), was not associated with this polymorphism.

Human Myospryn Polymorphism in Patients

Analysis of the interaction between the K2906N myospryn polymorphism and cardiac function in all subjects showed evidence that in a recessive model, this polymorphism is associated with the antero-septal wall thickness of the left ventricle (genotype AA: 10.85 mm, AC+CC: 10.02 mm, $p=0.0324$) as well as with A/E (genotype AA: 1.27, AC+CC: 1.09, $p=0.0278$). We further divided the subjects into two groups according to the antero-septal wall thickness for an association study. Subjects with antero-septal wall thickness greater than 10 mm were considered the case group with cardiac hypertrophy and were compared to the control group (all other subjects). In the association study of LV wall thickness, these groups differed significantly in K2906N polymorphism ($p=0.006$), sex ($p=0.003$), and HT ($p=0.003$); these differences were statistically estimated by the *t*-test with Bonferroni adjustments (Table 4).

Expressional Analysis of Myospryn in Heart

Finally, we examined expression of the myospryn gene in the heart. Northern blot analysis showed that human myospryn was mainly expressed in heart and muscle tissues (Fig. 2A), consistent with previous reports of mouse myospryn localization. Treatment of rat neonatal cardiac myocytes with angio-

tensin II (1 μmol/L) and phenylephrine (20 μmol/L) induced an increase in heart myospryn mRNA level (Fig. 2B). Moreover, continuous administration of angiotensin II (100 or 500 ng/kg/min) induced an increase in mouse heart myospryn mRNA level and induced cardiac hypertrophy, as confirmed by assessment of the myosin light chain (MLC)-2v (Fig. 2B). Recent studies have demonstrated that myospryn is a direct transcriptional target for myocyte enhancer factor 2A (MEF2A) in the heart and that myospryn mRNA level was increased approximately 3-fold in hearts overexpressing MEF2A (*17*). These results suggest that myospryn may mediate induction of cardiac hypertrophy, possibly by transducing stretch stimuli in striated muscle cells.

Discussion

Cardiac hypertrophy is an independent risk factor for morbidity and mortality, and the etiology of cardiac hypertrophy is multifactorial and may involve systemic factors such as volume or pressure overload, with the latter related to increased peripheral vasoconstriction (*22*, *23*). The present study showed that the novel sarcomeric protein myospryn is also associated with the development of LV hypertrophy.

Decentralized models of mechanotransduction propose that that mechanical stress applied at the cell surface is transmitted throughout the cell *via* the cytoskeleton. The term "tensegrity" has been applied to describe the transmission of mechanical forces from one part of a cell to another. This would theoretically allow the process of mechanotransduction to occur at a locus distant from the site of the applied strain (*24*, *25*). The tensegrity model of mechanotransduction is supported by a variety of different data, including the fact that, after application of a mechanical stimulus, isolated myocytes *in vitro* are able to respond with an increase in their gene expression, protein synthesis, and cell size (*26*, *27*). The ability to sense stretch does not necessarily depend on humoral or neural factors, but on an intact stretch sensor complex inside the cell. These and other data obtained using a variety of different agents and antagonists gave rise to the hypothesis that the whole cell is a mechanosensor (*28*). However, it is likely that some molecules or macromolecular structures have more important implications in mechanotransduction than others.

Previous studies have demonstrated that mutations in many sarcomeric protein-encoding genes can result in hypertrophic cardiomyopathy, an inherited predisposition towards increased ventricular wall thickening and a corresponding decrease in size of the ventricular cavity (*29–31*). Cytoskeletal proteins in the Z-disc of cardiac muscle, such as actin, desmin, and metavinculin, play important roles in stabilization of the sarcomere and in the transmission of force across the Z-disc (*9*, *32*). Indeed, recent studies have shown that myospryn localizes to the periphery of the Z-disc in the costamere, an elaborate protein network that links the sarcolemma to the Z-disc (*33*). These data suggest that myospryn may mediate the induction of cardiac hypertrophy or stiffness in response to pressure overload. This is supported by observations from the present study that a myospryn polymorphism was independently associated with increased LV cardiac hypertrophy in patients. Although in this study serum creatinine was also associated with K2906N polymorphism in a general population, we speculate that it may be associated with the change in skeletal muscle but not with urinary function, because myospryn expression was fairly restricted in heart and skeletal muscle and there was no difference in another urinary marker, BUN.

A limitation of the present work might be that we did not distinguish the patients taking different types of antihypertensive drugs. Thus, we cannot speculate on what kind of antihypertensive drug would be effective for myospryn-related cardiac hypertrophy. Moreover, it might be difficult to explain the discrepancy between antero-septal wall thickness and LV mass index in this study. A large-scale analysis is needed to clarify this in the future.

In conclusion, the present study demonstrated that a myospryn polymorphism was associated with LV diastolic dysfunction in hypertensive patients. Further study would be beneficial, both to characterize the mechanical stress–dependent signaling pathways linking myospryn to cardiac pathology and to identify therapeutic strategies to prevent the disease or stop its progression.

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