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Mutation of the phospholamban promoter associated with hypertrophic cardiomyopathy

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Abstract

Phospholamban is an endogenous inhibitor of sarcoplasmic reticulum calcium ATPase and plays a prime role in cardiac contractility and relaxation. Phospholamban may be a candidate gene responsible for cardiomyopathy. We investigated genome sequence of phospholamban in patients with cardiomyopathy. PCR-based direct sequence was performed for the promoter region and the whole coding region of phospholamban in 87 hypertrophic, 10 dilated, and 2 restricted cardiomyopathic patients. We found a heterozygous single nucleotide transition from A to G at −77-bp upstream of the transcription start site in the phospholamban promoter region of one patient with familial hypertrophic cardiomyopathy. This nucleotide change was not found in 296 control subjects. Using neonatal rat cardiomyocytes, the mutation, −77A → G, increased the phospholamban promoter activity. No nucleotide change in the phospholamban coding region was found in 99 patients with cardiomyopathy. We suspect that the mutation plays an important role in the development of hypertrophic cardiomyopathy.

Keywords: Cardiomyopathy; Hypertrophy; Calcium cycling; Sarcoplasmic reticulum; Gene mutation

Primary cardiomyopathy is a group of intrinsic disorders of the myocardium. To date, more than 18 genes have been identified as being responsible for cardiomyopathy [1,2]. Recently, mutations in the cardiac ryanodine receptor, a Ca^{2+} release channel in the sarcoplasmic reticulum (SR), have been identified as the cause of arrhythmogenic right ventricular dysplasia, a specific type of cardiomyopathy [3]. This is the first SR gene which causes cardiomyopathy.

The SR plays an integral role in the excitation–contraction coupling. Activity of the cardiac SR Ca^{2+} ATPase is the rate-determining factor of Ca^{2+} re-uptake into the SR and is regulated by phospholamban (PLN) that plays a prime role in cardiac contractility and relaxation [4]. Genetic complementation studies have revealed that inactivation of PLN can prevent the progression of cardiomyopathy [5,6]. In addition, mice harboring PLN mutations which result in gain-of-function [7] or cardiac-specific overexpression of PLN [8] display a cardiomyopathic phenotype. Thus, PLN may be a candidate gene responsible for cardiomyopathy. Here, we investigated the genome sequence of PLN in patients with cardiomyopathy.

Methods

Study population. All patients gave informed consent to the clinical and genetic study and the study was approved by the internal ethics

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committee of Tokyo Women's Medical University. The patients were evaluated by a detailed history, physical examination, 12-lead electrocardiography (ECG) and echocardiography. The clinical diagnosis of cardiomyopathy was based on echocardiographic findings. Individuals with another intrinsic cardiac or systemic disease capable of producing myocardial abnormalities were excluded. The study population consisted of 87 patients with hypertrophic cardiomyopathy (HMP), including 6 transition forms from HCM to dilated cardiomyopathy, 10 patients with dilated cardiomyopathy, and 2 patients with restricted cardiomyopathy.

Mutation screening. Mutation screening was performed on genomic DNA samples that were extracted from peripheral blood lymphocytes, as previously described [9], and the genomic structure of the human PLN gene was obtained from a previous report [10]. The human PLN gene consists of 2 exons separated by a single large intron. We prepared the following primer pairs: (1) 5′ tatttttctcataattaaaattcctgc 3′ and 5′ aaagtaagaattaccaaagtcagcg 3′ to amplify a 490-bp fragment of the promoter region (up to −342 bp upstream of the transcription start site) and the entire exon 1, (2) 5′ aacaatagtgctgaggaagatgaa3′ and 5′ ttgttttcctgtctgcatgg 3′ to amplify a 436-bp fragment of a part of exon 2 including the whole coding region of PLN. Amplified products were purified using the QIAquick PCR purification kit (Qiagen) and were directly sequenced with the ABI-PRISM dye-terminator cycle sequencing kit using a ABI310 genetic analyzer (ABI Biosystems). A control group of 296 healthy and unrelated subjects was used to exclude the possibility of the detected mutations being DNA polymorphisms.

Restriction enzyme assay. The single nucleotide transition, −77A → G, abolished a *Taq*I site in the PCR products. The digested fragments by *Taq*I were confirmed by size fractionation on 2% agarose gels.

Promoter activity assay. The 490-bp fragment containing the nucleotide transition, −77A → G, or the wild type of the promoter region was subcloned into the promoter-less firefly luciferase expression vector, pGL3-Basic (Promega). Neonatal rat ventricular myocytes were prepared as described previously [11]. After 24-h incubation with serum-free medium, they were transiently co-transfected with 300 ng of each PLN luciferase test plasmid and 75 ng of phRL-TK control plasmid (Promega), using FuGene 6 (Roche). The luciferase activity was measured 24 h after transfection with Dual-Luciferase Reagents (Promega).

Thapsigargin (10 nM) or 0.0001% DMSO was added for the last 45 h of the 48 h incubation. Data are expressed as means ± SEM. Differences were considered significant at $P < 0.05$ (two-way ANOVA).

Results

Mutation screening

We found a heterozygous single nucleotide transition, −77A → G, in the PLN promoter region of one patient with familial HCM (#p1397) (Fig. 1A). The mutation was confirmed by detecting a non-digested fragment by *Taq*I (Fig. 1B). This nucleotide transition, −77A → G, was not found in 296 control subjects (592 alleles). No nucleotide change in the PLN coding region was found in 99 patients with cardiomyopathy.

To exclude the possibility that mutations of β-myosin heavy chain, troponin T, and myosin binding protein C genes, which are the most frequent causal genes of HCM, accompanied with the mutation, −77A → G, PCR-based direct sequencing was performed in the patient according to a previous report [12]. No mutation was found in the regions of frequent occurrence of mutations in these genes.

The clinical profile of the patient with mutation, −77A → G

The patient with mutation, −77A → G, was female and presented the first episode of paroxysmal atrial fibrillation at the age of 56 years. Physical examination showed a systolic ejection murmur (2/6). The cardiothoracic ratio was 59% at chest X-ray. The 12-lead ECG showed left ventricular hypertrophy and ST-T wave abnormality. Echocardiography showed that the thickness of septal and posterior wall of left ventricle was 30 and 13 mm, respectively. The diastolic and systolic dimension of left ventricle was 48 and 27 mm, respectively. No significant mitral regurgitation nor left ventricular outflow obstruction was found. Her father who was diagnosed with cardiomyopathy died at the age of 82 years and an elder brother at the age of 62 years was also diagnosed as having HCM. The family history indicated a late onset type of HCM.

Promoter activity assay

When neonatal rat cardiomyocytes were transiently transfected with luciferase reporters under control of the wild type or mutant (−77A → G) human PLN promoter, we found that the point mutation, −77A → G, resulted in a 1.5-fold increase in transcriptional activity, compared to the wild-type promoter. In the situation of SR stress induced by thapsigargin, the PLN promoter activity of the mutant is significantly enhanced (Fig. 2).

Discussion

We found a novel mutation in the PLN promoter region which is associated with HCM and did not find the nucleotide transition in 296 control Japanese subjects (592 alleles). The mutation site is within the region that plays a critical role in expression of the PLN gene [11,13] and is close to a very conserved CCAAT element (−84 to −80), on which the nuclear protein NF-Y binds to regulate the PLN promoter activity. Although the mutation site is not well conserved when compared with dog, cat, rabbit, mouse, and rat, Montovani described that the specific additional nucleotides (C/G, A/G, **G**, A/C, G on the 3′ side of CCAAT box) are required for efficient binding of NF-Y/CP1 with CCAAT box [14]. Therefore, we hypothesized that the mutation, −77A → G, increased the promoter activity. Indeed, using neonatal rat cardiomyocytes, the mutation, −77A → G, increased PLN promoter activity. Thus, we speculate that an in-

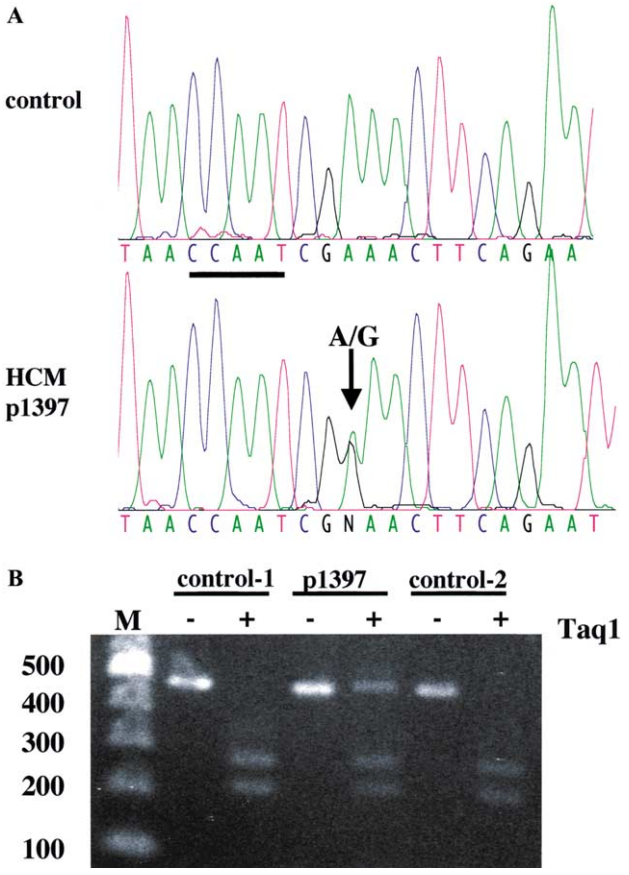

Fig. 1. (A) Genomic DNA sequence analysis of the PLN promoter region in wild type (upper panel) and a patient with HCM (#p1397, lower panel). Underlining indicates the CCAAT element (−84 to −80). (B) Restriction enzyme assay. Since the A → G transition abolished a *Taq*I site in the PCR products, a non-digested fragment was detected in the patient with mutation on 2% agarose gels.

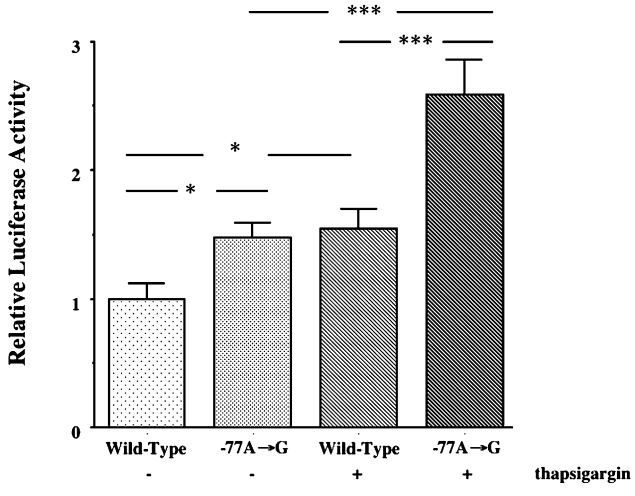

Fig. 2. Effect of the mutation, −77A → G, on human PLN promoter. Transcriptional activity was defined as a ratio of firefly luciferase activity to Renilla luciferase activity in the same cells, and normalized with the mean transcriptional activity of the wild-type PLN promoter. The values were expressed as means ± SEM of four independent experiments. * $P < 0.01$, *** $P < 0.001$.

crease in PLN promoter activity induces the higher PLN expression, and that the increase in PLN expression modulates the cardiac function for a long period and, eventually, results in cardiac remodeling. Dash et al. [8] demonstrated that transgenic mice, in which the cardiac PLN expression is four times higher than in non-transgenic mice, develop a late-onset type of cardiomyopathy. This mouse model is likely to fit our case, although it should be noted that the contribution of SERCA/PLN on Ca^{2+} removal from cytosole is much higher in the murine heart than in the human heart. Unfortunately, we could not examine the level of the PLN expression in the patient as well as co-segregation of the mutant with other affected family members, since the patient was inaccessible anymore. For these reasons, at this moment, we do not conclude that the single nucleotide transition, −77A → G, is a HCM-causal mutation. Nevertheless, we think that the mutation is not a mere polymorphism and that the increased PLN expression caused by a mutation in the promoter region plays an important role in the development of HCM in human.

Although we did not find any mutation in PLN coding region in our patients, two independent investigators recently found that PLN missense mutations cause human dilated cardiomyopathy [15,16]. Since different mutations in several sarcomeric proteins such as β-myosin heavy chain and troponin T cause dilated and hypertrophic cardiomyopathies [1,2], PLN is also likely to be associated with both dilated and hypertrophic cardiomyopathies.

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