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Sensitivity and Specificity of Denaturing HPLC to Detect *MYBPC3* Gene Mutations in Hypertrophic Cardiomyopathy

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Denaturing high-performance liquid chromatography (DHPLC), which has recently been developed as an automated method to detect mutations, is at least ten times less expensive than the direct sequencing method; however, its sensitivity and specificity for cardiac myosin-binding protein C (*MYBPC3*) gene mutations in hypertrophic cardiomyopathy have not been reported yet. A mutation analysis of exons 1 to 35 of *MYBPC3* gene from 20 Japanese patients with hypertrophic cardiomyopathy was performed using DHPLC and direct sequencing. Compared to direct sequencing, the sensitivity and specificity of DHPLC were 87.5 % and 97.42 %, respectively. Its positive and negative predictive values were 41.18 % and 99.74 %. The positive and negative likelihood ratios were 33.95 and 0.13, and the prevalence was 2.02 %. DHPLC showed high sensitivity and specificity for detecting *MYBPC3* gene mutations in hypertrophic cardiomyopathy. The use of this complementary sequencing method should reduce the cost of detection of *MYBPC3* gene mutations, and could be used to screen patients with hypertrophic cardiomyopathy.

Key words: cardiac myosin-binding protein C gene; denaturing high-performance liquid chromatography; hypertrophic cardiomyopathy; mutation detection

Genotyping of sarcomeric genes is currently used to identify mutations in patients with familial hypertrophic cardiomyopathy; hence, it is essential to have an automatic, inexpensive, non-hazardous, highly sensitive and specific method for genetic analysis. Hypertrophic cardiomyopathy is inherited as a Mendelian-autosomal dominant trait and is caused by mutations in any of the 13 genes encoding proteins of the cardiac sarcomere and the non-cardiac sarcomeric protein (Seidman et al., 2001; Richard et al., 2006). In several studies, mutations of the cardiac myosin-binding protein C gene (*MYBPC3*) were found to be one of the two most common genetic causes of familial hypertrophic cardiomyopathy (Jääskeläinen et al., 2002; Erdmann et al., 2003; Mörner et al., 2003; Richard et al., 2003). To date, more than 150 mutations, consisting of missense, deletion/insertion and splice site abnormalities, have been identified in *MYBPC3*.

Direct sequencing is the gold standard method for the detection of gene mutations, but it is laborious, time consuming and expensive (Suzuki et al., 2009). Alternative mutation screening techniques may play a role in both candidate gene analysis and

Abbreviations: DHPLC, denaturing high-performance liquid chromatography; *MYBPC3*, cardiac myosin-binding protein C gene

diagnosis. Ideally, a method to screen mutation in new samples should be sensitive, non-hazardous, relatively inexpensive and fully or at least semi-automatic to minimize the time required for the test, labor and cost. Denaturing high-performance liquid chromatography (DHPLC) has recently been developed as an automated method to detect mutations, and it is at least 10 times less expensive than the direct sequencing method (O'Donovan et al., 1998). Therefore, it potentially overcomes the drawbacks of the direct sequencing method. However, the sensitivity and specificity of DHPLC to detect *MYBPC3* gene mutations in hypertrophic cardiomyopathy have not been reported yet. In this study, we analyze *MYBPC3* gene in patients with hypertrophic cardiomyopathy and asses the sensitivity and specificity of DHPLC by comparing them with those of the direct sequencing method.

Subjects and Methods

The subjects were 20 Japanese patients diagnosed as having primary hypertrophic cardiomyopathy at Tottori University Hospital. Briefly, clinical diagnostic criteria were adults with maximum myocardium wall thickness of > 13 mm determined by echocardiography in the absence of any other confounding diagnosis. Genomic DNA was extracted from peripheral white blood cells using the standard phenol-chloroform procedure. Exons 1 to 35 of the *MYBPC3* gene were amplified by PCR using primers designed according to the published genomic sequences of *MYBPC3* (Carrier et al., 1997). PCR products were run in 1% agarose gel, stained with ethidium bromide and visualized employing a UV transilluminator.

DHPLC analysis was performed using the Transgenomic WAVE system (Transgenomic, Omaha, NE) to detect genetic variants. To obtain heteroduplexes with the WAVE system, the prediction of temperature and gradient condition was determined using the Stanford Genome Technology Center software (http://insertion.stanford.edu/melt.html) and the WAVEMAKER software. Three melting temperatures were chosen from the predictions. Three types of samples were chosen for DHPLC, i.e., wild type as a control, samples from hypertrophic cardiomyopathy patients, and a mix of wild type and patient samples. The heteroduplex peak resolve was influenced by the specific nucleotide mismatch present and the melting characteristics of the surrounding bases. Elution profiles that differed from wild type indicated the presence of mutation or polymorphism. Normal samples were recruited for control (wild type) amplicon of DHPLC analysis from 5 healthy Japanese. The sequencing was performed with the Abi Prism 373 DNA Sequencer (Applied Biosystems, Foster City, CA) according the manufacturer's instructions. This study has been approved by the Ethical Committee of Faculty of Medicine, Tottori University.

The diagnostic test was based on a 2X2 contingency table analysis to compare DHPLC and the sequencing method. Since the sample amplicon of DHPLC and sequencing was a DNA fragment from each exon of the *MYBPC3* gene, the sample unit which was used for statistical analysis was each exon of *MYBPC3*. The estimated number of mutations of the *MYBPC3* gene in hypertrophic cardiomyopathy is 1 mutation in each patient. Thirty three fragments of PCR product were obtained from 35 exons of *MYBPC3* (Fig. 1A). Exon 2 was amplified in 2 separated fragments. Three separated fragments were obtained from 3 of 2 short tandem exons. They were exon 14–15, exon 19–20 and exon 34–35. Using the formula of single proportion, 12 positive signals of DNA amplicon were required in DHPLC, and the minimum total number of DNA amplicons was 369.

Results and Discussion

All *MYBPC3* exons were successfully amplified by PCR at 60˚C melting temperature (Fig. 1A). DHPLC was performed for *MYBPC3* gene DNA from all 20 patients and 5 normal subjects. Waves with 2 or more peaks in the amplicon from patient samples indicated a heterozygous mutation or

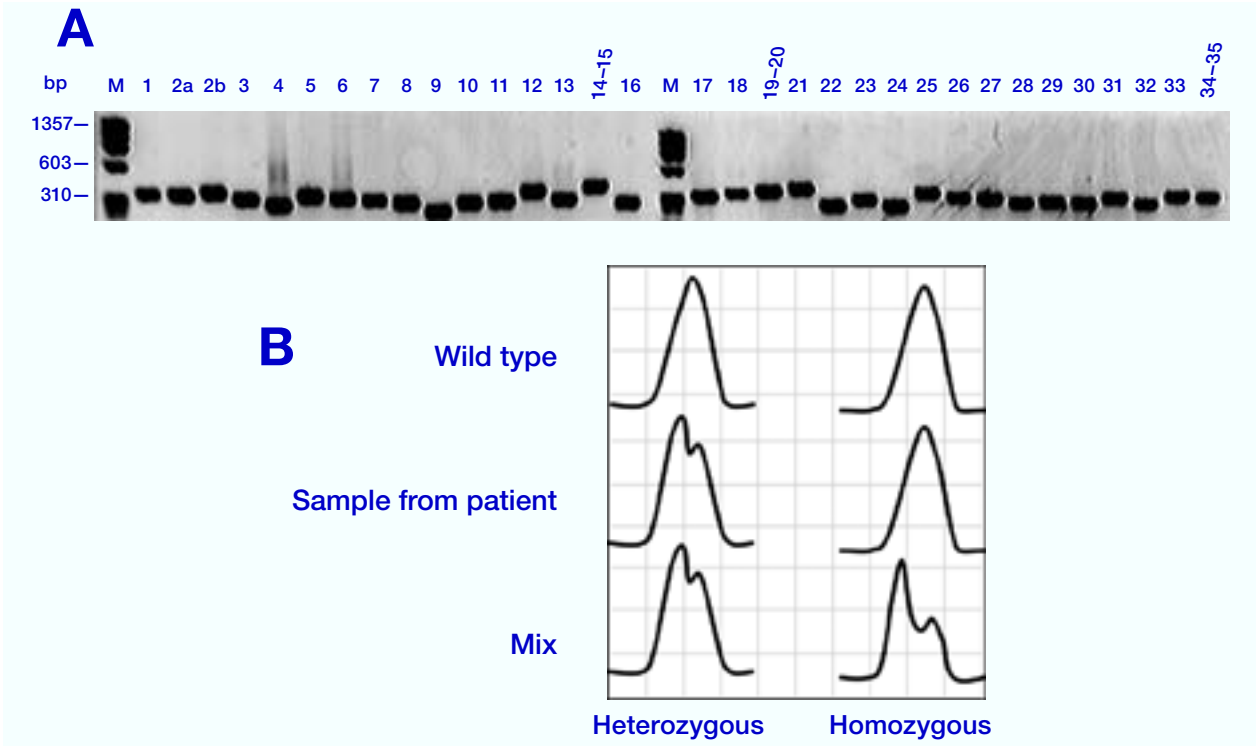

Fig. 1.

A: PCR amplification product of *MYBPC3* gene from exon 1 to exon 35. Exon 2 was amplified using 2 different primer sets; each of exons 14 and 15, 19 and 20, and 35 and 36 were amplified using one primer set. All *MYBPC3* exons were successfully amplified by PCR at 60˚C melting temperature.

B: An example of DHPLC wave analysis from the same segment of amplified DNA. Double-peak waves in the amplicon from a patient DNA sample indicated a heterozygous mutation or polymorphism, whereas, a wave with two or more peaks in the mix amplicon indicated a homozygous mutation or polymorphism.

DHPLC, denaturing high-performance liquid chromatography; M, molecular weight marker.

polymorphism, whereas, in the mix amplicon they indicated a homozygous mutation or polymorphism (Fig. 1B). We found more than one peak of the DHPLC wave at exon 5 of samples from both control subjects and patients indicating the probability of polymorphism.

Sequencing was performed for all DNA samples from the patients. Consistent with the DHPLC result, we found a polymorphism in exon 5 of *MYBPC3* gene in samples from patients and normal subjects, and therefore, we excluded exon 5 from the analysis of sensitivity and specificity of DHPLC versus sequencing. Three hundred sixty nine DHPLC waves out of 660 were reliable for the analysis of sensitivity and specificity of DHPLC versus sequencing. As shown in Table 1, one false negative and 10 false positive were identified by DHPLC. The sensitivity and specificity of DHPLC were 87.5 % and 97.42 %, respectively. In

Table 1. Sensitivity and specificity of DHPLC versus sequencing

	Sequencing (+)	Sequencing (–)	Total
DHPLC (+)	7	10	17
DHPLC (–)	1	378	379
Total	8	388	396

DHPLC, denaturing high-performance liquid chromatography.

Sensitivity = 87.5%, specificity = 97.42%, positive predictive value = 41.18%, negative predictive value = 99.74%, positive likelihood ratio = 33.95, negative likelihood ratio = 0.13, prevalence = 2.02%.

addition, its positive and negative predictive values were 41.18 % and 99.74 %, the positive and negative likelihood ratios were 33.95 and 0.13, and the prevalence was 2.02 %.

Since a practical application of DHPLC genetic analysis would be screening to reduce unnecessary sequencing, it is important that its sensitivity be the highest obtainable. In several studies to detect mutations in various genes, the sensitivity of DHPLC consistently exceeded 96 % (Underhill et al., 1997; Liu et al., 1997-1998; Wagner et al., 1999a, 1999b; Xiao et al., 2001). The ability of DHPLC to detect a mutation depends upon the formation of heteroduplexes (Xiao et al., 2001; Rudolph et al., 2002); the mixed amplicon should be tested using various ratios. For optimal mutation detection, it was important to evaluate the PCR reaction and running profile. In this study, we used Taq DNA polymerase for the PCR reaction and sequencing. The sensitivity and specificity of DHPLC may be increased by using other polymerases which have higher fidelity. This warrants further study.

Identification of mutations in sarcomeric genes is meaningful for patients with hypertrophic cardiomyopathy. Once a mutation is identified in a patient, screening of all family members is recommended, particularly for those who have symptoms of cardiac disease. For this reason, it is important to establish an inexpensive method that improves screening of *MYBPC3* gene mutations in hypertrophic cardiomyopathy.

Mutation analysis using sequencing is expensive because each exon is sequenced in both directions. The cost per run, even in subsidized laboratories, is about US $10 to $25, excluding the costs of DNA isolation, PCR reagents and labor/time (O'Donovan et al., 1998; Xiao et al., 2001; Suzuki et al., 2004). Any mutation screening must be technically simple, use inexpensive reagents, and be highly specific and highly sensitive. Therefore, a mutation screening method may contribute to reduce the number of unnecessary sequencing tests. DHPLC is a relatively new method to detect mutations. It is run automatically and it is relatively inexpensive. Each amplicon of DHPLC is screened for about US $1.5 (Wagner et al., 1999). This inexpensive method can become affordable worldwide, particularly for early genetic diagnosis or gene mutation screening.

Finally, we conclude that because of the high sensitivity and specificity of DHPLC regarding detection of *MYBPC3* gene mutations in hypertrophic cardiomyopathy, it should become a useful mutation screening method for hypertrophic cardiomyopathy.

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