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Short Communication

High-resolution melting curve analysis for genotyping of common SNP in *MTHFR* gene using fixed-cell suspension

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ABSTRACT

Genetic variation in *MTHFR* might explain the interindividual differences in both therapeutic and toxic responses to the treatment of cancer and rheumatoid arthritis with methotrexate, and can be involved in the sensitivity of developing diseases like cancer and congenital anomalies. We investigated the common sequence variation, C677T, in the *MTHFR* gene in fixed-cell specimens archived after chromosomal analysis using a novel gene scanning method based on post PCR analysis of high-resolution melting curves (HRM). These fixed specimens were stored after routine chromosomal analysis for 1 year at −20 °C in a 3:1 methanol:acetic acid solution. The method revealed a distinct pattern between homozygous and heterozygous alleles. Sensitivity and specificity of the HRM based method were comparable to that obtained by a hybridization probe. While the success rate for genotyping of a common SNP in MTHFR was similar to the hybridization probe approach, the HRM based method was more cost-effective and had a shorter turnaround time.

1. Introduction

The most common form of human genetic variation is caused by dimorphic single nucleotide polymorphisms (SNPs), which are important candidates as causal variants of traits. SNPs might contribute either directly or indirectly to an individual's predisposition to complex diseases such as cardiovascular diseases, cancer, obesity, diabetes, psychiatric illness, and inflammatory diseases [1]. Modern, genetic research requires methods that allow rapid, reliable, accurate, and cost-effective SNP genotyping across a broad range of samples. Currently, there are a large number of genotyping technologies available. The two common methods used in many studies generally employ one of two mechanisms for allelic discrimination, namely allele-specific hybridization [2] or primer extension [3]. Melting techniques that use fluorescently labeled oligonucleotide probes to genotype the short segments of PCR product were introduced many years ago. Measuring the melting temperature between the probe and the PCR product allows detection of the different types of alleles. However, this method provided information only if the sequence variant was within the detection probe. In contrast to the probe methods, entire PCR products can be melted in the presence of DNA binding dyes such as SYBR® Green I [4] and that can differentiate the double-stranded DNA from the single-stranded DNA by the change in fluorescence intensity. Sequence variants were inferred from changes in the melting temperature of the PCR product. Recently, techniques for high-resolution melting curve analysis have appeared along with the introduction of a new family of LCGreen® dyes [5]. Melting analysis with these labeled dyes is well documented on many platforms [6]. In the current study, we performed a systematic comparison of melting analysis by using probe hybridization and LCGreen® to study a common SNP found in the MTHFR gene. We also tested the possibility of using cells fixed in 3:1 methanol:acetic acid solution as a source of genomic DNA for genetics testing. These fixed cells are routinely archived in many cytogenetic labs after the chromosome analysis or fluorescence in situ hybridization (FISH) studies, and we have shown that such specimens can be used as a source of genomic DNA for identification of sequence variation.

2. Materials and methods

2.1. DNA samples

Fifty-six unrelated Thai individuals with acute lymphoblastic leukemia (ALL) were included in this study. Samples for each

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individual were sent to our lab for routine cytogenetic analysis. Total genomic DNA was isolated from 3:1 methanol:acetic acid fixed cells using the Cell and Tissue Kit (Gentra systems, Minnesta, USA). DNA extraction was performed as recommended by the manufacturer. These fixed-cell suspensions were archived after a routine chromosome analysis from a short term bone marrow or peripheral blood culture and stored for 1 year at −20 °C in the 3:1 methanol:acetic acid solution. The study was approved by the Ethics Committee of Chulabhorn Research Institute. These samples were then used in all methods utilized in this study. Three control DNA samples which had known *C677T MTHFR* polymorphisms were also used in each experiment.

2.2. Genotyping by hybridization probe analysis

Genotyping of the *C677T MTHFR* polymorphism was performed on a 480 LightCycler® instrument (Roche Diagnostics, Mannheim Germany) using a commercial real-time assay (LightMix®, Roche Diagnostics). A 10 μl reaction mixture was prepared in a 96-well PCR plate containing 65 ng genomic DNA, 1 μl for each probe (LightMix® for the detection of human *MTHFR* C677T, TIB MOLBIOL GmbH, Berlin Germany), 5 μl 2× LightCycler® 480 Probes Master (Roche Diagnostics, Mannheim Germany), 1.5 μl Dnase-free water (PCR-grade), and processed according to the manufacturer's instructions (Roche Diagnostics). Real-time PCR of MTHFR C677T was performed by an initial denaturation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 5 s, 55 °C for 10 s, and 72 °C 15 s. In order to determine the melting points after the amplification phase, a melting curve analysis was performed at 95 °C for 20 s and at 40 °C for 20 s, followed by a slow heating at the rate of 0.2 °C/s until reaching 85 °C. The polymorphism was determined to have a specific melting point (T_m) of 55 °C for the mutant and 62.5 °C for the wild type.

2.3. High-resolution melting curve analysis

The 250 ng of sample DNA was first amplified by real-time PCR in the presence of saturating DNA dye contained in the LightCycler® 480 high-resolution melting master. Two primer sets were evaluated. The first set was the primers which were previously reported for genotyping of C677T by the RFLP method [7]. Their sequences are, 5′TGAAGGAGAAGGTGTCTGCGGGA3′ (forward) and 5′AGGACGGTGCGGTGAGAGTG 3′(reverse). The second set, was designed in our laboratory by the Primer3 program [8], and has the sequences 5′CTTTGAGGCTGACCTGAAGC3′ (forward) and 5′GAAAAGCTGCGTGATGATGA3′ (reverse). The 20 μl amplification reaction was prepared in 96-well plates PCR containing 5 μl genomic DNA, 0.5 μl of 1 μM of each primer, 10 μl 2× LightCycler® 480 High-Resolution Master mix, 2 μl H_2O (PCR-grade), and 2 μl of 2.5 mM $MgCl_2$. The PCR reactions were modified as follows: for 10 min initial denaturation at 95 °C, followed by 45 cycles at 95 °C for 10 s, 64 °C for 15 s, and 72 °C for 8 s. After PCR, the samples were heated to 95 °C for 1 min and then cooled to 40 °C before melting. Then the PCR products were heated from 65 °C to 95 °C, at the rate of 1 °C per second. The fluorescence data were subsequently visualized using normalization, temperature-shifting, and difference plotting [5], and then analyzed by the automated grouping functionality provided by the LightCycler® 480 Gene scanning software. Analysis of the genotypes was performed blind, without knowledge of the known genotype.

For the novel primers, the PCR amplification was performed in similar conditions as the former experiment, except the annealing temperature was substituted with the touchdown protocol, ranging from 64 °C to 58 °C.

2.4. RFLP

For C677T genotype analysis, the *MTHFR* gene was amplified with the following primers, 5′TGAAGGAGAAGGTGTCTGCGGGA3′ (forward) and 5′AGGACGGTGCGGTGAGAGTG3′ (reverse). The MTHFR genotype was determined by the method described by Frosst et al. [7]. The PCR products were then digested with the HinfI restriction enzyme. The genotypes were obtained by visual inspection of the 3% agarose gels.

3. Results

All of the 56 samples were successfully genotyped with these two methods: probe hybridization and HRM. The reproducibility for genotype calls was 100% in both methods when performing inter- (3 controls of known MTHFR genotype samples were run in all experiments) and intra-runs (4 replicate samples). The genotypes of MTHFR C/C, MTHFR C/T, and MTHFR T/T were present in 63.33%, 33.33%, and 3.33% of the samples, respectively.

The PCR products from the first primer set [7] were 198 base pairs, while the PCR products from newly designed primer set were only 73 base pairs. By using the first primer set for MTHFR, there was 8.9% (5 out of 56 samples) non-concordance in the genotyping of both hybridization probe and HRM methods. Restriction length polymorphism analysis (RFLP) was used to confirm the result of these 5 samples (Fig. 3). The result of the RFLP was similar to those using gene scanning by LightMix® and the shorter amplicons (Table 1). With the newly design primers, 100% concordance of the genotype results was obtained and the genotype could be better distinguished (Figs. 1–3).

4. Discussion

Methylenetetrahydrofolate reductase (MTHFR) is an essential enzyme in the metabolism of folate and DNA methylation. The presence of polymorphisms that reduce the activity of MTHFR has been linked to the development of many diseases such as leukemia [9], breast cancer [10], and colon cancer [11]. Currently, candidate-gene-based association studies of MTHFR are still a common approach used in disease-causing gene identification research. The MTHFR C677T allele has been important in pharmacological responsiveness of MTX sensitivity in treatment of many diseases like acute lymphoblastic leukemia (ALL) [12], rheumatoid arthritis [13] and ovarian cancer [14]. Many methods for genotyping of *MTHFR* were introduced, including RFLP [7], real-time PCR with fluorescence probe [15], the TaqMan assay [16], minisequencing [17] and mass spectrometry [18]. In this study, we have described and successfully utilized a genotyping technology, namely the high-resolution melting curve (HRM) for the detection of the C677T polymorphisms in the *MTHFR* gene. The HRM method combines the advantages of using melting curve techniques and without the requirement of allele-specific primers or probes to target specific variants. Only two primers are required for identification of the allele change. Additionally, using a short amplicon for genotyping maximizes the melting temperature (T_m) difference

Table 1
Non-concordance of genotyping results in acute leukemia patients by using a probe hybridization method and the HRM method

Patient ID	Method			
	Probe Hybridization	HRM1 (193 bp)	HRM2 (73 bp)	RFLP
BM 188-49	CT	UNKNOWN	CT	CT
BM 249-49	CT	TT	CT	CT
BM 1104-49	CT	CC	CT	CT
BM 1112-49	CC	CT	CC	CC
BM 12-50	CC	CT	CC	CC

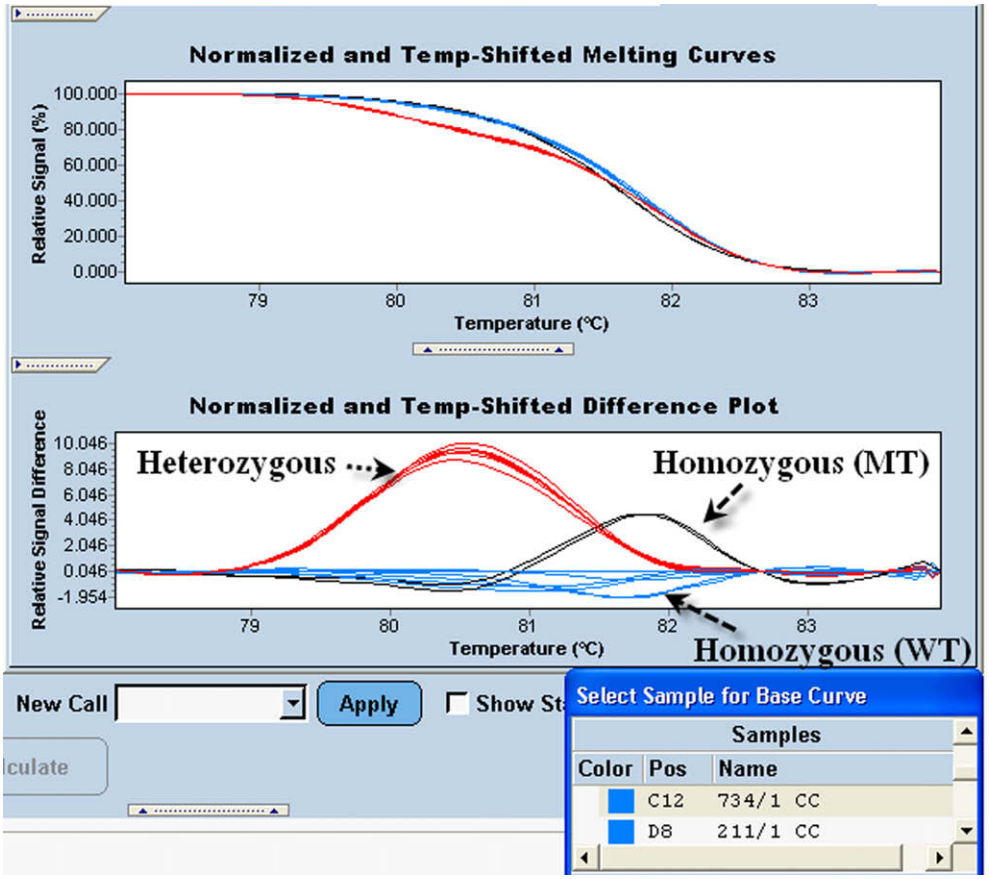

Fig. 1. High-resolution melting analysis of a 73-bp-fragment derived from the MTHFR gene containing a C to T polymorphism in the presence of high-resolution melting dye. The difference plots were created by using the wild type CC as a base line. Homozygous (wild-type) is shown in blue, heterozygous in red and homozygous (mutant) is in black.

between wild-type and homozygous mutant genotypes, and this was observed as the T_m differences among genotypes increased and as the amplicon size decreased, allowing better differentiation [19]. The differences among genotypes were more obvious when the amplicons were shorter. However, amplicon size was varied from experiment to experiment to assess size suitability. In our study, the better results were obtained by using shorter fragments, which may also be due to the quality of the DNA. The DNA extracted from fixed-cell suspensions in this study, mostly degraded into small fragments (data not shown).

In conditions where the heterozygous variants, could be correctly differentiated, but the homozygous mutant (TT) and homozygous wild type (CC) couldn't be exactly discriminated, another method, called spiking of wild type DNA could be used. This would be performed by adding known homozygous genotypes to unknown samples, thereby creating a pseudo-heteroduplex, and allowing the melting curve to separate in both homozygous genotypes [19,20].

Comparing the costs, instrument run time, genotype success rate and the versatility of these two methods, the use of the HRM procedure with shorter amplicons is currently the best method to genotype the *MTHFR* C677T SNP. The HRM procedure was less expensive in terms of cost per sample and had shorter running time. The cost for reagents for genotyping by HRM was low because only PCR primers and single high-resolution dyes are needed. The running time used in HRM is about 1 h, which is less than the hybridization probe method. The success rate of genotyping by the hybridization probe was higher than HRM method when the amount and quality of the starting DNA were poor. Therefore, a standardized DNA amount is required of the HRM method.

In some instances, obtaining additional blood samples from the patients for further studies was not possible. Therefore, we have shown that fixed-cell suspensions archived after routine chromosome studies can now be used as genomic DNA source for re-evaluating probe hybridization or HRM without the need for additional blood samples from the previously studied individuals.

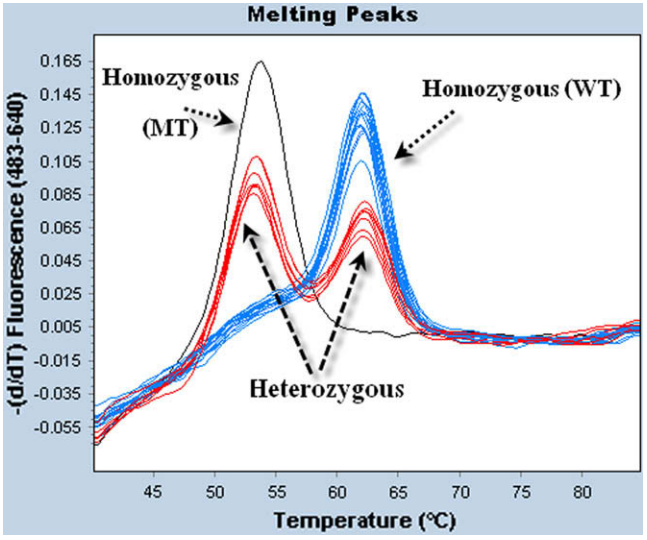

Fig. 2. The melting curve patterns of the hybridization probe method for C677T MTHFR gene polymorphism. Melting curve for homozygous (wild-type) is shown in blue, heterozygous in red, with 2 distinct peaks, and homozygous (mutant) in black.

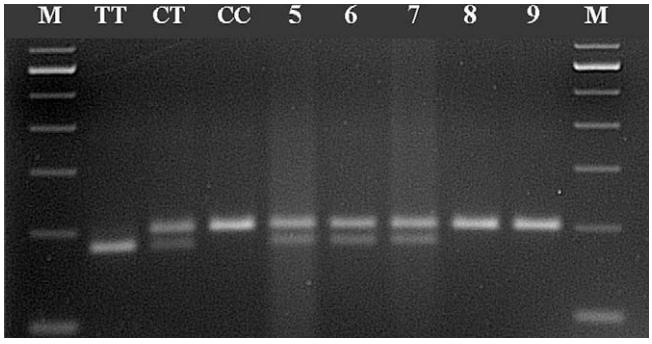

Fig. 3. PCR-RFLP analysis results for *MTHFR* C677T polymorphisms. Lane 1 and 10 are 100 bp DNA marker. Lane 2 3 and 4 are control for 677TT genotype, heterozygous 677CT genotype and homozygous wild type 677CC genotype, respectively. Lane 5 is BM 188/1 heterozygous 677CT genotype. Lane 6 is BM 249/1 heterozygous 677CT genotype. Lane 7 is BM 1104/1 heterozygous 677CT genotype. Lane 8 is BM 1112/1 homozygous 677CC genotype. Lane 9 is BM 12-50/1 homozygous wild type 677CC genotype.

In conclusion, the HRM method is an effective genotyping technique which can be used for revealing both unknown and known mutations in interesting genes.

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