



Original Article

Rapid screening for mutations associated with malignant hyperthermia using high-resolution melting curve analysis

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ABSTRACT

Objectives: The diagnosis of malignant hyperthermia (MH) is based on clinical signs or laboratory testing. The gold standard laboratory test is the *in vitro* contracture test, although it is invasive, expensive, and only performed at specialized centers. Genetic diagnosis is another option, although direct mutation screening is a laborious task. Therefore, we evaluated whether high-resolution melting (HRM) curve analysis could be used as a rapid screening tool to target MH-associated mutations. **Materials and Methods:** The feasibility of HRM analysis was evaluated using plasmids that were constructed by cloning wild-type or mutated versions of the ryanodine receptor 1 (*RYR1*) gene into the pCR2.1 plasmid. We obtained engineered plasmids and patient DNA extracted from blood samples with known wild-type or mutated sequences that are associated with MH. Amplicon lengths were kept relatively short (<250 bp) to improve discrimination between the engineered and patient plasmids. Real-time polymerase chain reaction (PCR) cycling and HRM analysis of the engineered plasmids and patient DNA were performed using the LightCycler 480 System (Roche). **Results:** The HRM results were clearly different from those obtained using real-time PCR. Furthermore, the HRM analysis provided sufficient resolution to identify two single-nucleotide variants in the tested *RYR1* exons. **Conclusion:** We conclude that HRM analysis can provide high resolution for identifying single-nucleotide variants in *RYR1*, which might be useful for predicting the risk of MH in the preanesthesia setting.

KEYWORDS: Biomarker, High-resolution melting analysis, Malignant hyperthermia, Polymerase chain reaction

INTRODUCTION

Malignant hyperthermia (MH) is an unusual but life-threatening pharmacogenetic disorder of the skeletal muscle, which involves calcium ion release from the sarcoplasmic reticulum. Susceptible individuals have a hypermetabolic response to depolarizing muscle relaxants and volatile anesthetics, as well as exercise- or heat-related stress in rare cases [1]. The classic signs of MH include tachycardia, tachypnea, muscle rigidity, increased carbon dioxide production, increased oxygen consumption, hyperthermia, acidosis, hyperkalemia, rhabdomyolysis, and arrhythmia. From a genetic perspective, MH is inherited as an autosomal dominant trait with variable expression in humans, although most cases involve a defect in the ryanodine receptor, which is encoded by the ryanodine receptor 1 (*RYR1*) gene located on chromosome 19q13.1 [2].

The diagnosis of MH is based on clinical signs or laboratory testing. The gold standard laboratory test is

currently the *in vitro* contracture test (IVCT), which is based on the contracture of muscle fibers in the presence of halothane or caffeine. However, the IVCT is expensive, only performed at specialized centers, and invasive because it requires a biopsy sample. A less invasive alternative is DNA analysis, which can identify pathogenic variants in *RYR1*, *CACNA1S*, or *STAC3*. Unfortunately, DNA analysis based on direct sequencing would involve a significant workload, as the *RYR1* gene includes 106 exons [3].


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High-resolution melting (HRM) curve analysis is a highly automatable method for detecting heteroduplex DNA fragments. The exon of interest is amplified through quantitative polymerase chain reaction (PCR), and a fluorescent signal is only produced if the probe binds to double-stranded DNA (dsDNA). The reaction temperature (T_m) is increased in small steps at the end of the PCR, which causes the dsDNA to separate into single strands at a specific T_m and leads to a sharp decrease in fluorescence [4]. The LightCycler® 480 System offers a user-friendly format that can perform the real-time PCR and HRM analysis in <1 h on a single instrument. Furthermore, the LightCycler® 480 Gene Scanning Software can be used to analyze and differentiate between melting curves based on the difference plot technique, which can be used to differentiate between wild-type and mutant samples based on the fluorescence from each sample [5]. However, HRM curve analysis has two important limitations. First, amplicon length influences the sensitivity and specificity of HRM curve analysis, and an amplicon length of <300 base pairs (bps) is recommended [6]. Second, different heterozygous samples may produce similar curves, even if the curves are clearly different from those of homozygous samples [7]. Nevertheless, given the burden associated with direct DNA analysis of all 106 exons in *RYR1*, we aimed to evaluate whether HRM analysis might be useful for rapidly identifying genetic variants that are linked to HM using genomic DNA samples with wild-type or mutant *RYR1*.

MATERIALS AND METHODS

Patients

The DNA samples for this study had been collected as part of a previous study regarding MH. As part of that study, all patients provided informed consent for the research use of their DNA samples. That study's protocol was approved by the ethical committee of Chang Gung Memorial Hospital (August 24, 2009; approval no. 98-2370C). All data were analyzed at the Tzu Chi Medical Center, Hualien.

Primers

The *RYR1* exon primers were designed using LightCycler Probe Design Software 2.0 (Roche) [Table 1]. To ensure adequately short amplicons for HRM, the maximum length was set to 250 bp in order to ensure that the analyses were sensitive and specific. We also used the original primers from the previous study to amplify exons 14–15 and exons 51–53.

Polymerase chain reaction

The DNA samples were obtained from either standard plasmids or genomic DNA. The reactions were performed using a total volume of 20 μ L, which contained 100 ng of DNA, 10 μ M of forward and reverse primers for each target exon, 10 mM dNTPs, 0.125 μ L of Taq polymerase, and 10X buffer. The cycling program was started at 95°C and maintained for 7 min as a “hot-start” process, which was followed by 35 cycles of 10 s at 95°C and 30 s at 60°C, and then a hold at 60°C for 1 min. The synthesis of appropriately sized products was confirmed via electrophoresis.

Denaturing high-performance liquid chromatography

Before the denaturing high-performance liquid chromatography (DHPLC) analysis, the PCR products were denatured at 95°C for 3 min and gradually reannealed by decreasing the sample temperature (T_m) to 65°C in decrements of $-1^\circ\text{C}/\text{min}$. Mutations were detected based on the formation of heteroduplexes after combining the PCR products from the patient with a normal control. The initial column T_m s and acetonitrile gradient were based on the Stanford DHPLC Melt program [8].

Construction of plasmids

After the desired exon was confirmed, the PCR product was purified. We added a 3'-end adenosine overhang to the PCR product using Taq DNA polymerases. Finally, the PCR product was ligated to the pCR2.1 plasmid using the TA-cloning method.

Sequencing

The DNA sequence analysis was performed by a sequencing company.

Real-time polymerase chain reaction and high-resolution melting analysis

The real-time PCR and HRM analysis of the engineered plasmids and genomic DNA samples were performed using the LightCycler® 480 System (Roche). Each run consisted of 5 samples, which included 1 blank sample, 2 wild-type dsDNA samples (homozygous), 1 mutant genomic dsDNA sample (heterozygous), and 1 patient sample. The real-time PCR was performed using SYBR® Green dye, with an amplification protocol that involved 35 cycles of 10 s at 95°C and 30 s at 60°C. The PCR products were then melted in the same test tube by slowly increasing the T_m from 60°C to 95°C, and the release of SYBR® Green dye was continuously monitored in 0.3°C increments to create the melting curve.

The HRM was performed using the HRM Master reagent (Roche), with 45 cycles of 10 s at 95°C, 15 s at 60°C, and 10 s at 72°C. The PCR products were then melted in the same test tube by slowly increasing the T_m from 65°C to 95°C. Data were continuously monitored in 0.04°C increments to create the melting curve. Differentiation of the melting curves was then performed using three simple steps. First, the LightCycler® 480 Gene Scanning Software was used to normalize the raw melting curve data by setting the premelt and postmelt signals of all samples to equal values. The software then shifted the normalized curves along the T_m axis to match the point where the dsDNA in each sample became completely denatured. The differences in the melting curves' shapes were then evaluated by subtracting the shifted normalized curves from a base curve. The final plot (a “difference plot”) allowed samples to be clustered into groups with similar melting curve shapes, as minor differences in curve shape and melting T_m became obvious through this process.

RESULTS

We initially verified the mutations using cDNA and DHPLC. Based on our previous findings, genomic DNA from stored samples were evaluated for mutations in exons 2, 6, 8, 11,

13, 14–15, 39, 44, 45, 46, 51–53, 67, 91, 95, 101, and 102. Figure 1 shows that we were able to successfully amplify 18 exons in *RYR1* hotspots for the present study. The PCR products were purified, screened using DHPLC, and then sequenced. Heteroduplex formation was observed in exons 14–15 using samples from patients 1–3 and in exons 51–53 using samples from patients 1 and 2. The sequencing results were compatible with the heteroduplex formation that was observed during DHPLC. A single-nucleotide variant was detected in exons 14–15, which involved TCG → TCA/G at residue 1695. Another single-nucleotide variant was detected in exons 51–53, which involved GAT → GAT/C at residue 8227 [Figure 2].

Construction of heterozygous and homozygous standard plasmids

Figure 3 shows the construction of heterozygous and homozygous standard plasmids. The PCR products based on genomic variants (A/G in *RYR1* exons 14–15 and T/C in exons 51–53) were first purified from the electrophoresis gel and then inserted into the pCR2.1 plasmid, which was then examined after EcoRI digestion. Direct sequencing was used to confirm that the plasmids contained the A/G variant in *RYR1* exons 14–15 or the T/C variant in exons 51–53. These confirmed plasmids were then used as the heterozygous standard plasmids. The pCR2.1-based plasmids were then transformed into TOPO 10 cells to express the heterozygous variants. Plasmids expressed in TOPO 10 cells were also subjected to sequencing to identify plasmids that contained A/A and G/G at residue 1695 (exons 14–15) or C/C and T/T

at residue 8227 (exons 51–53), which were then used as the homozygous standard plasmids.

Using high-resolution melting analysis to differentiate between G/G, A/A, and A/G in ryanodine receptor 1 exons 14–15

We evaluated whether HRM analysis could differentiate between G/G, A/A, and A/G in *RYR1* exons 14–15, and the real-time PCR and HRM protocols are shown in Figure 4. The tests included a negative control, plasmids containing the A/A, G/G, and A/G variants, as well as a patient sample [Figure 5a]. The primers for the real-time PCR are shown in Table 1 and there were no significant differences in the reaction conditions for each sample [Figure 5b]. The results for the PCR reaction efficiency and the real-time PCR melting curve analysis are shown in Figure 5c. Dissociation of the dsDNA was observed at 60–95°C [Figure 5d] and processing the raw data (change in fluorescence level per unit change in T_m, dF/dT) revealed a dissociation peak in the curve at 81°C [Figure 5d]. The melting curves for each standard plasmid (A/A, G/G, and A/G) had distinct slopes and peak times, and the melting curve of the patient’s cDNA sample was very similar to the melting curve for the A/G plasmid standard.

Using high-resolution melting analysis to differentiate between C/C, T/T, and C/T in ryanodine receptor 1 exons 51–53

We also evaluated whether HRM analysis could differentiate between C/C, T/T, and C/T in *RYR1* exons 51–53. The tests included a negative control, plasmids

Table 1: Primer sequences designed to amplify exons in hotspot regions

Exon	Primers (5'→3')	°C	Exon	Primers (5'→3')	°C
14-15 (470 bp)	F: TGAATTCGTGAATCCAAGAAGACAAG R: TGCCCAGAATGAGAGGTGAAGC	58	2 (438 bp)	F: GTATCTCAAGGAGTTGTCAGGAGCA R: CTCAGGTAGCCCTCTTAGCCTTA	63
51-53 (842 bp)	F: GATGATTGCAGTGTGTGAGTTTGAG R: ATCCACCTAGATGCAACATGTGA	58	8 (298 bp)	F: CCAAACCTCAGCCCTCAGGTTC R: GGAATCTGGTCCCTAATCCTACTCA	63
11 (345 bp)	F: CTGCACTCTGCAGTCCCTCA R: GTACAGTGGCATGATCACCAGCTCA	58	39 (447 bp)	F: CTTCCACATTGTTCTGGTCAAGG R: CAGAAGTTGGGAAGGGAATGTTAT	67
6 (251 bp)	F: GGGAAAGAGCATTCTGGGAAGC R: CAAACCCATGGTGAGAAGATGG	60	45 (516 bp)	F: AGAGCGTGGAGGAGAACGCCAAT R: GTTGTGTCCCCAACATTGCTAGTC	67
13 (378 bp)	F: AGTGACGTTGCGGCAGTTAGC R: CTTCTCAGACCCTACTTCCCCAGGT	60	46 (248 bp)	F: GTGTGGTAAGGGAGGGAGCAGA R: TCCCCAGCATCACTCCTTCG	67
44 (486 bp)	F: CTGGTGTACCCTAGAGGTGTTG R: GTTTTCTCAGGCGGTTCTCAC	60	67 (488 bp)	F: CTGCTAGGTGGAGATGCTGTTT R: AAGAAACCAGGAGGAAGAGTCAGAA	67
95 (465 bp)	F: ATCTGGTATGGTCCCAGTCCAATCT R: CCTCTGTCCCAACCACTTTGAGG	60	101 (374 bp)	F: AGGTAGAGCCACAGGGACTGAAC R: CAACTCCTGGACTCAAGTAATCGTC	67
91 (955 bp)	F: CTGACGGCGCCATCCTGT R: AGCCAGTTCTCTCTGTGTGTGT	60	102 (376 bp)	F: GGCTGTCTCAGTCGTTACCATGTCT R: GCGAGAGGTAGAGATGGGGTATGAA	67

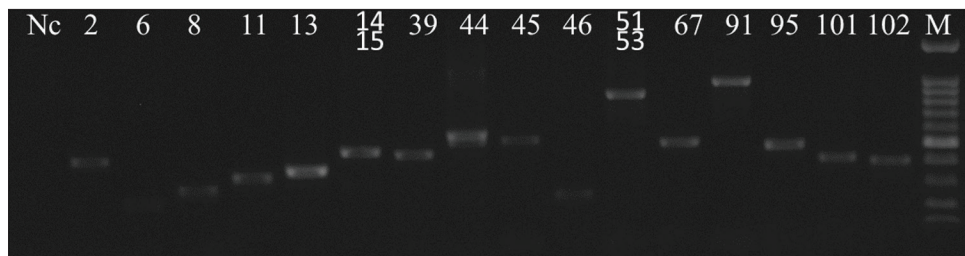


Figure 1: Gel electrophoresis revealed successful amplification of 18 exons in hotspot regions

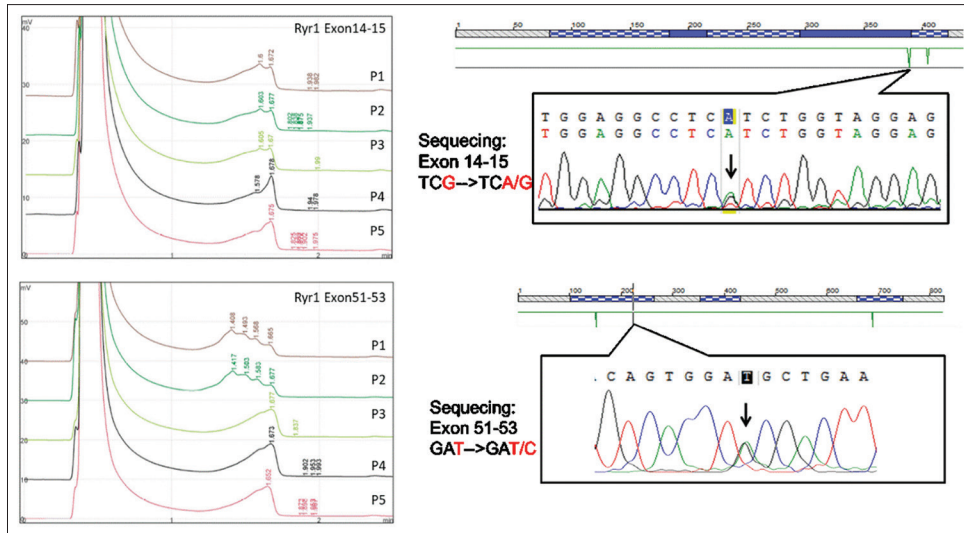


Figure 2: Heteroduplex formation was observed in samples from patients 1–3 (*RYR1* exons 14–15) and in samples from patients 1 and 2 (*RYR1* exons 51–53) through denaturing high-performance liquid chromatography

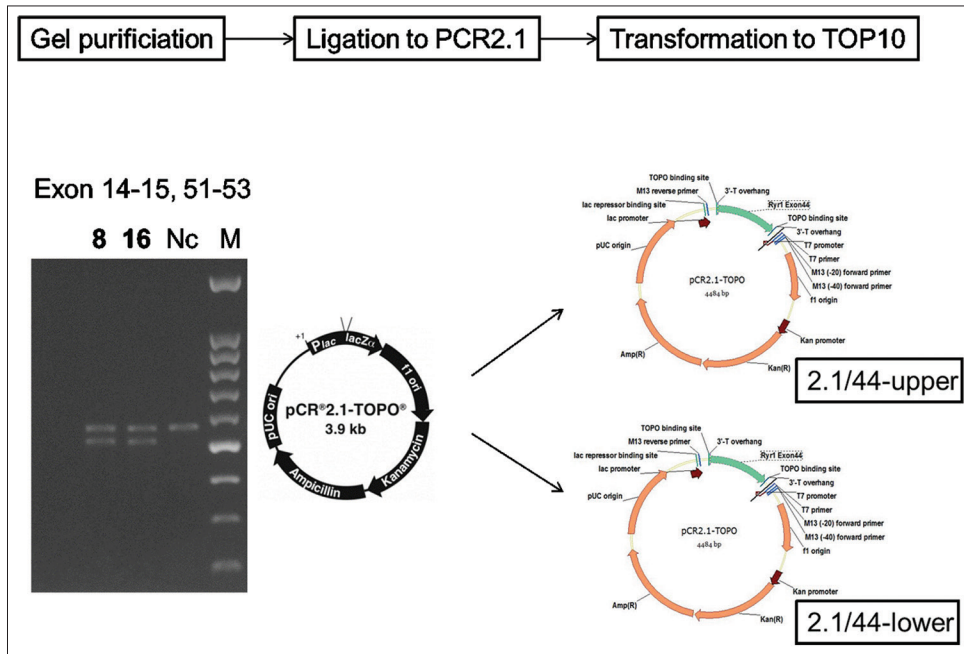


Figure 3: The process for constructing the testing plasmid

containing the C/C, T/T, and C/T variants, as well as a patient sample [Figure 6a]. The real-time PCR primers are shown in Table 1 and the reaction conditions are shown in Figure 4. The results for the PCR reaction efficiency and the real-time PCR melting curve analysis are shown in Figure 6b. There were no significant differences in the reaction conditions for real-time PCR and HRM for each sample [Figure 6c]. Dissociation of the dsDNA was observed at 60°C–95°C [Figure 6a] and processing the raw data revealed a dissociation peak in the curve at 81°C [Figure 6d]. The melting curves for each standard plasmid (C/C, T/T, and C/T) had distinct slopes and peak times, and the melting curve of the patient’s cDNA sample was very similar to the melting curve for the C/T plasmid standard.

Normalized and temperature-shifted difference plots increased the ability to identify single-nucleotide variants

The HRM melting curves were then analyzed based on normalized and T_m-shifted difference plots with the HRM Master Mix (Roche). The single-nucleotide variant in exons 14–15 was used to test the efficacy of this method by comparing two heterozygous samples (a patient sample and the plasmid with A/G at residue 1695) and two homozygous samples (plasmids with A/A and G/A at residue 1695) [Figure 5a]. The normalized curve for each standard plasmid (A/A, G/G, or A/G) had clearly distinct curve patterns that could be differentiated based on visual inspection [Figure 7a]. The patient’s cDNA curve was

most similar to the curve of the heterozygous standard plasmid, which suggested that this method could identify a single-nucleotide variant in *RYR1* exons 14–15. The method was also tested using the single-nucleotide variant in exons 51–53 [Figure 7b] by comparing three heterozygous samples (two patient samples and the plasmid with T/C at residue 8227) and four homozygous samples (two normal patient samples and two plasmids with T/T and C/C at residue 8227). The normalized curves for each standard plasmid (C/C, T/T, and C/T) were clearly distinct and could be differentiated based on visual inspection. The patient’s cDNA curve was most similar to the curve of the heterozygous standard plasmid, which suggested that this method could identify a single-nucleotide variant in *RYR1* exons 51–53.

DISCUSSION

There is currently no standard preanesthesia test to screen for MH before routine surgery, which is related to current genetic testing methods being expensive and time-consuming. The present study aimed to evaluate whether HRM could serve as a convenient screening tool to identify patients with

a high risk of MH, based on *RYR1* mutations and their family history. Our results indicate that HRM provided sufficient ability to identify single-nucleotide variants in *RYR1* exons based on difference plots, which suggests that HRM might be useful as a preanesthesia test to screen for MH before surgery.

The gold standard test for diagnosing MH is the IVCT, which involves exposing living muscle fiber bundles (from a biopsy sample) separately to halothane and caffeine, based on a strict protocol that was developed based on the observations of Kalow *et al.* [9] and Ellis *et al.* [10] in the 1970s. The IVCT provides 99% sensitivity and 93.6% specificity for diagnosing MH [11], although the testing process is invasive, expensive, and limited to a few specialized centers. Therefore, it would be useful to identify a simpler, less-invasive, and inexpensive method for diagnosing MH, which has drawn attention to genetic testing as a possible alternative.

The human *RYR1* gene contains >15,000 bps [12] and it would be laborious, tedious, and expensive to perform traditional PCR and direct sequencing to identify single-nucleotide polymorphisms that are associated with MH. The more recently developed DHPLC strategy is also not convenient as a preanesthesia laboratory test. Furthermore, there are >40 known *RYR1* mutations in patients, with other studies investigating additional candidate genes and site-specific mutations. Thankfully, there is evidence that related mutations are likely clustered in hotspot regions, which suggests that a high-throughput screening method focused on *RYR1* hotspots would be practical. While examining the hotspots located in the RYR1 3D protein folding map, it is interesting that all the hotspots are located at the inner face of the channel. The RyR1 channels with those hotspot mutations have significantly lowered threshold for activation by Ca²⁺ and increased threshold for inactivation by Ca²⁺ [13]. In this

95 °C	7 min	} 35 cycle
95 °C	10 sec	
60 °C	30 sec	
Plate read		
60 °C	1 min	
Melting Curve range 60~95°C, recording per 0.3°C		

Figure 4: The real-time PCR and high-resolution melting protocol. PCR: Polymerase chain reaction

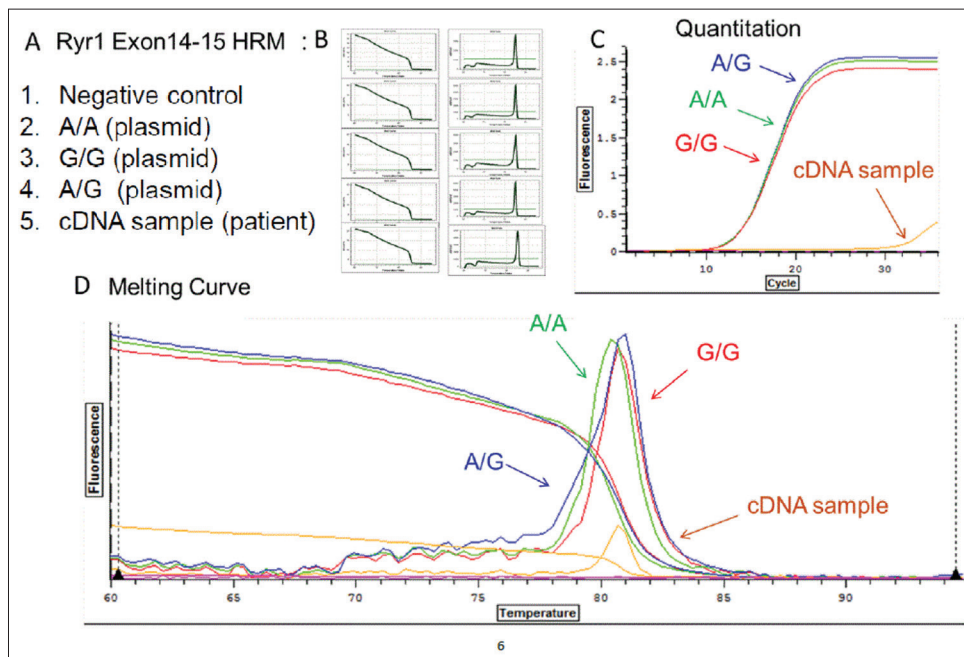


Figure 5: The ability of high-resolution melting analysis to differentiate between G/G, A/A, and A/G in RYR1 (exons 14–15). (a) The samples used for high-resolution melting analysis. (b) The melting curve raw data of each sample (left) and the negative first derivatives (-dF/dT), revealing melting temperatures at peaks. (c) The real-time PCR amplification curves by SYBR® Green fluorescence. (d) The melting curve of the patient’s cDNA sample was similar to the melting curve of the A/G plasmid standard

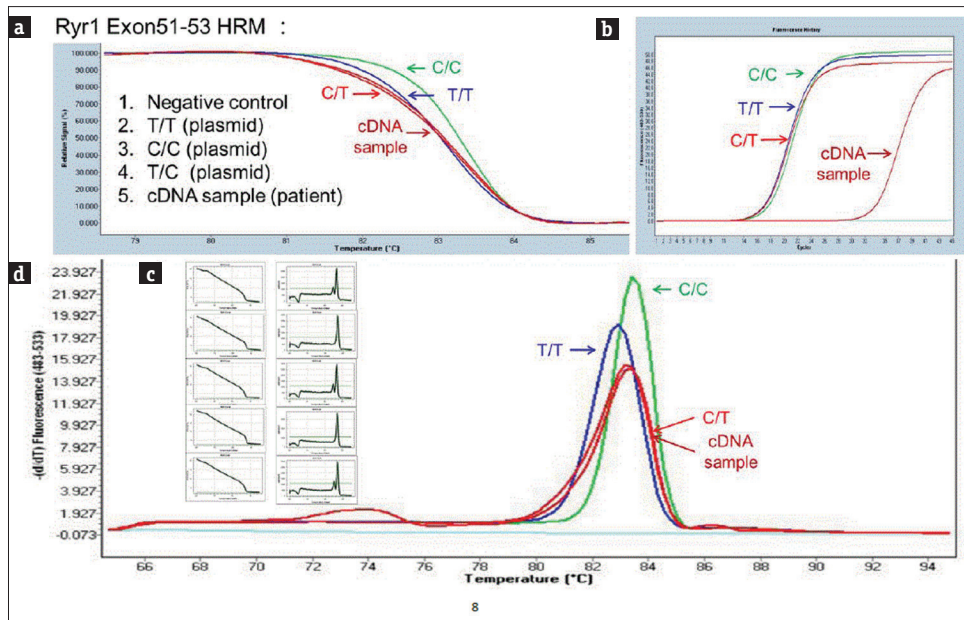


Figure 6: The ability of high-resolution melting analysis to differentiate between C/C, T/T, and C/T in RYR1 (exons 51–53). (a) HRM analysis using different samples included a negative control, plasmids containing the C/C, T/T, and C/T variants and a patient sample. (b) The real-time PCR amplification curves by SYBR® Green fluorescence. (c) The melting curve raw data of each samples (left) and the negative first derivatives (-dF/dT), revealing melting temperatures at peaks. (d) The melting curve of the patient's cDNA sample was similar to the melting curve of the C/T plasmid standard

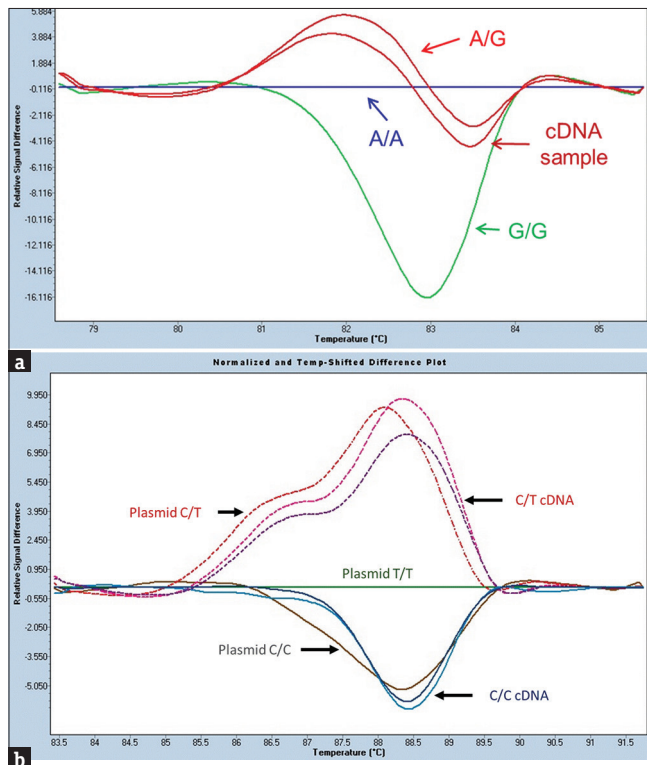


Figure 7: High-resolution melting curve analysis by normalizing and temperature-shifted difference plot using the HRM master (Roche). (a) The patient's cDNA curve was similar to the curve of the heterozygous standard plasmid, and a single-nucleotide variant could be detected in RYR1 (exons 14–15). (b) The patient's cDNA curve was similar to the curve of the heterozygous standard plasmid, and a single-nucleotide variant could be detected in RYR1 (exons 51–53). HRM: High-resolution melting, RYR1: Ryanodine receptor 1

when used to identifying various inherited diseases [14]. This test identifies nucleotide variations through heteroduplex formation between wild-type and mutant DNA strands and has been used to successfully identify RYR1 mutations in patients with MH [15]. Therefore, we used DHPLC before the HRM to identify candidate exons (based on heteroduplex formation) among the 106 exons in the RYR1 gene. This strategy might also be used to create additional engineered plasmids for a rapid screening kit to diagnose or predict the risk of MH.

In the present study, the HRM analysis was performed using the LightCycler 480 system to evaluate plasmids containing short fragments of wild-type or mutated RYR1. Comparing the melting curves of the patient samples and standard plasmids allowed us to identify single-nucleotide variants based on the shapes of the melting curves, which confirmed that a single-nucleotide variant in a fixed position within a small replicon could be reliably identified using this method. Using plasmids with known mutations in RYR1 hotspots might permit prediction of MH risk based on a 1-h assay using a single 48-well plate, and it is possible that this strategy could be extended to other genes that are potentially related to MH in the future.

As an alternative to direct sequencing, HRM analysis is a homogeneous post-PCR method that uses the same reaction tube, does not require separate processing, and eliminates the need for labeled probes that are specific for single-nucleotide polymorphisms [16]. Furthermore, HRM analysis can reliably distinguish between test and control sample sequences that differ by as little as a single nucleotide [17]. This method relies on a specific dye to detect the heteroduplex dsDNA (e.g., LightCycler® 480 HRM dye), which can be used in saturating concentrations without adversely affecting the preceding PCR and provides a fluorescence signal that is at least eight times

context, DHPLC is a useful and efficient method for mutation screening in a large number of sequences, with high sensitivity

stronger than the signal of LCGreen Plus [18]. These factors are what prompted us to use the HRM Master reagent in this study. We also focused on relatively short amplicons and identified single-nucleotide variants using only two unlabeled primers, which may not require extensive optimization or an expensive HRM machine, as the analyses could in theory be performed using a standard PCR machine. However, the LightCycler® 480 system offers 96-well or 384-well plate formats and completes the assay within 1 h. Moreover, the LightCycler® 480 Scanning Software can create normalized and T_m -shifted difference plots to highlight minor differences in curve shape, which makes this machine very convenient. Finally, the real-time PCR preceding the HRM analysis can provide a useful quality control step, as late or poor amplification can reduce the validity of HRM analysis [19].

Although the present study only evaluated one mutation in *RYR1*, the results suggest that developing plasmids containing many MH-associated mutations might allow for rapid screening to predict the preanesthesia risk of MH. Using our methods, an assay could be developed to cover all *RYR1* hotspots and potentially other genes that are associated with MH, such as *RYR3*. A rapid and high-throughput screening method might be possible to predict the risk of MH in this setting if a sufficient number of engineered plasmids can be validated. However, there were some limitations of genetic screening. First, the sensitivity of genetic testing is relatively low compared to functional testing (IVCT). Genetic screening may be a complete alternative to the functional testing but cannot replace the role of function testing [20]. Second, genotype positive individuals may not be phenotype positive [21]. Overall, the genetic screening provided the risk stratification and the chance to early detection.

CONCLUSION

HRM analysis provided high resolution for differentiating between wild-type and mutated *RYR1* based on single-nucleotide variants, which might be useful for guiding the prediction of MH in the preanesthesia setting. Using normalized and T_m -shifted difference plots in this strategy also increased its ability to identify specific single-nucleotide variants in patient samples.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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