Discovering Rare Variants by Use of Melting Temperature Shifts Seen in Melting Curve Analysis

Two reports in this issue of Clinical Chemistry describe novel mutations in thrombophilia genes (1,2). The authors of the reports describe variants detected by the identification of a melting temperature \((T_m)\) different from the expected \(T_m\) of the target mutation and from the \(T_m\) of the wild-type sequence. The variants reported in this issue are A20218G in the prothrombin (factor II) gene and C1690T in the factor V gene. These cases add to the reported variants identified by fluorescent hybridization probes and melting analysis on the LightCycler. In addition to factor II and V variants, variants have been discovered similarly in genes coding for MTHFR and HFE (3,4).

Melting analyses, as performed on the LightCycler or similar instruments, can distinguish variants that lie within the region of the target probe. Differences in base composition, nucleotide position, and nearest-neighbor environments all affect \(T_m\). These differences are detected by monitoring fluorescence during an increase in temperature. Melting is visualized by a loss of fluorescence as the probes dissociate from the template. Differences in \(T_m\) from the expected \(T_m\) for wild-type and target mutations are indicative of a nontarget (a variant other than the mutation the assay was designed to detect) mutation or variant.

In contrast, assays that use restriction enzymes, allele-specific amplification, or hybridization probes at a single detection temperature may not detect a variant or may show an indeterminate genotype (3,4). With these 3 types of assays, when one allele carries the variant and the opposite allele is a wild type, the variant allele may be undetected, and the genotype would be reported as wild type. If the variant is in conjunction with a target mutation on the opposite allele, the genotype could be reported as a homozygous mutant, rather than a mutant/variant compound heterozygote. The melting analysis avoids these pitfalls.

Variants with \(T_m\) shifts of 2–5 °C are easily visualized, as was the case of the prothrombin A20218G case reported by Tag et al. (1). However, if the base change produces a sequence with a temperature stability similar to that of the target mutation, the \(T_m\) shift may be subtle. Other variants, as discussed by Mahadevan and Benson (2), could be mistaken for a Leiden mutation if careful quality-control measures are not followed. By tracking \(T_m\)s over time, a \(T_m\) range can be established for each allele. However, \(T_m\)s can vary between samples as a result of several factors, including salt or DNA concentrations. Calculating the difference in \(T_m\)s between the wild-type and mutant peaks (\(\Delta T_m\)) in heterozygous samples is more precise than relying on \(T_m\) alone. \(\Delta T_m\)s are less affected by sample-to-sample differences because both alleles are affected equally. The \(T_m\) variation of any specific allele typically ranges from 0.2 to 0.5 °C, whereas \(\Delta T_m\) variation is often 0.1 °C or less (5). Ninety-five percent confidence intervals (2 SDs) of \(\Delta T_m\) are ±0.2 °C. For factor V and MTHFR assays, we have identified variants with \(\Delta T_m\)s of ~0.4 °C. In the report of Mahadevan and Benson (2), the variant they found was readily identified by both the \(\Delta T_m\) and the \(T_m\).

The excellent precision exhibited in these assays led us to hope that different variants may be identified by \(\Delta T_m\) alone, eliminating the need for sequencing. After confirming an HFE variant T189C (6) by sequencing more than a dozen samples, we now identify this variant by \(\Delta T_m\) alone. By contrast, for the prothrombin gene, we have found 3 different variants with \(\Delta T_m\)s that were within 0.5 °C of each other and had overlapping 95% confidence intervals. We therefore routinely sequence all prothrombin samples with \(T_m\)s to confirm the variant. The cost of sequencing is not an important factor because these variants constitute a small proportion of tested samples.

For samples that appear homozygous (wild type or mutant), identifying variants is more difficult because \(\Delta T_m\)s cannot apply. In these cases, \(T_m\) alone, or in conjunction with the shape of the curve (a shoulder or broad peak), can often suggest a variant.

The frequencies of these variants differ among target genes. Factor V variants are rare, with 1 detected in ~17 000 samples (1 in 34 000 chromosomes). In addition to the reported factor V C1690T variant (which prematurely terminates the protein chain), we have also seen A1696G (7), which produces an isoleucine-to-valine change in the activated protein C (APC)-resistance pocket (8); 1690delC (7), which causes a frameshift; and G1689A (no amino acid change) (9). Prothrombin variants C20209T (10), A20207C (5), A20218G (1), and C20221T (11) are detected in ~1 in 1660 samples (1 in 3300 chromosomes). Of these variants, C20209T accounts for nearly 85%. Of the HFE variants (1 in 1000 samples) T189C (6), a silent mutation, is present in 88% of the variants. HFE variants C842A (Thr281Lys) (4) and A854C (Glu285Ala) at the C282Y locus and G197A (Arg66His; H63D locus) (6) were each found once. MTHFR has several common variants (3); C685G (I622Val) has an allele frequency of 1 in 4700, and G679A (Asp223Asn) has an allele frequency of 1 in 3300 in our sample set. Overall, MTHFR variants are detected in 1 of 750 samples.

A common misconception is that rare variants are on different chromosomes. We have found several cases in which a rare variant is on the same chromosome (i.e., in cis) as a target mutation (4). The phase can be determined by testing parents to confirm co-inheritance of the mutation and the variant from one parent. Alternatively, a variant seen with a homozygous mutation confirms that one chromosome contains both a mutation and the variant.

After identifying a variant, determining its consequence can be challenging. A variant that causes a frameshift or a stop codon is reported as causative or suspected causative, as is the case for the factor V C1690T variant. However, as Mahadevan and Benson (2) point out, a nonsense mutation at this position could have clinical consequences different from those of the Leiden mutation. Silent mutations such as...
the HFE variant T189C are usually considered insignificant, with the caveat that they could be involved with splice sites or DNA/protein binding sites. A missense mutation is more difficult. For example, the factor V A1696G changes an amino acid and is within the APC-cleavage pocket. However, the isoleucine-to-valine change is considered a conservative change. The prothrombin variants all are within the 3′ untranslated region of the gene, adding to the challenge in determining their relationship with the patient’s clinical condition. The rarity of these variants makes disease association studies difficult. Family concordance studies are feasible, but the family must be sufficiently large to show statistical significance. Studies are further complicated when the penetrance is low and the disease (such as venous thrombosis) is common.

We turn then to functional assays to assess the effect of the sequence alteration on production of the protein. In vitro methods use cell lines transfected with prothrombin “minigenes” to measure gene expression (12). Ceelie et al. (13) from the Leiden University Medical Center developed a functional assay for prothrombin mutations. In this assay, genomic DNA is amplified by PCR and cloned downstream from a luciferase cDNA in pGL3-basic. They showed that the G20210A mutation increases mRNA and that protein synthesis is consistent with clinical studies. The same assay showed that the A20218G and A20207C mutations did not increase mRNA or protein. The results suggested instead that the mutated sites may decrease polyadenylation efficiency. Although slightly lower than for the wild type, mRNA concentrations seen with the variants were not significantly different from those seen with the wild-type sequence; thus these variants are unlikely to contribute to clinical symptoms (14). In contrast, Danckwardt et al. (15) showed that C20221T is likely to be clinically significant, with effects similar to those of the G20210A mutation.

A question that arises when a variant other than the target mutation is detected is whether to report it. Some may argue that only mutations listed in the published assay claims should be reported, particularly if the clinical consequences of a variant are unknown. Others argue that all findings, including those detected coincidentally with the target mutation, should be reported. Reporting variants as clinically inconsequential is also debatable because today’s polymorphism may be a “modifier” tomorrow. In this case, a result may be reported based on a possible future discovery rather than current evidence.

Mutation discovery has been further investigated with high-resolution melting. Precise temperature control improves differentiation of DNA sequences. High-resolution melting with a general DNA dye such as LC Green™ has been used for genotyping with either unlabeled probes (16) or direct amplicon melting (7). The variants detected by fluorescence resonance energy transfer (FRET) hybridization probes are also differentiated by these methods, and many more heterozygotes can be differentiated (6). High-resolution melting has also been used for mutation scanning to identify any mutation within the amplicon.

As is often the case, knowledge is advanced as research findings are introduced into the clinical laboratory. In some cases, such as in the factor V gene, the rare variants were found only by testing thousands of samples. Common variants have also been found that could confound results. The genetics community is well aware that rare variants may interfere with any molecular assay. With the dynamic melting analysis on the LightCycler, variants not detected by commonly used methods in the clinical laboratory were detected and distinguished from the target mutation. In addition to finding potentially disease-causing mutations, knowing about these rare variants and their frequencies also can help predict their effect in assay design and method assessment.

References


Elaine Lyon
Pathology Department
University of Utah School of Medicine
ARUP Laboratories
Salt Lake City, UT

Address for correspondence: ARUP Laboratories, Medical Directors, 500 Chipeta Way, Salt Lake City, UT 84108.

DOI: 10.1373/clinchem.2005.051177