Detection of the Hereditary Hemochromatosis Gene Mutation by Real-Time Fluorescence Polymerase Chain Reaction and Peptide Nucleic Acid Clamping

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Received February 10, 1998

Hereditary hemochromatosis (HH), an iron overload disease, is the most common known inheritable disease. The most prevalent form of HH is believed to be the result of a single base-pair mutation. We describe a rapid homogeneous mutation analysis method that does not require post-polymerase chain reaction (PCR) manipulations. This method is a marriage of three emerging technologies: rapid cycling PCR thermal cyclers, peptide nucleic acid (PNA) probes, and a new double-stranded DNA-selective fluorescent dye, Sybr Green I. The LightCycler is a rapid thermal cycler that fluorometrically monitors real-time formation of amplicon with Sybr Green I. PNAs are DNA mimics that are more sensitive to mismatches than DNA probes, and will not serve as primers for DNA polymerases. PNA probes were designed to compete with PCR primers hybridizing to the HH mutation site. Fully complemented PNA probes at an 18:1 ratio over DNA primers with a mismatch result in suppression of amplicon formation. Conversely, PNA probes with a mismatch will not impair the binding of a complementary primer, culminating in amplicon formation. A LightCycler-based rapid genetic assay has been developed to distinguish HH patients from HH carriers and normal individuals using PNA clamping technology.

Hereditary hemochromatosis (HH)2 is an iron overload disease that afflicts 1.5 million Americans, and is the most prevalent identified inheritable disease. HH often goes undiagnosed, or misdiagnosed, and is frequently the root cause of many liver diseases, cardiovascular diseases, arthritis, and diabetes. HH is treatable by therapeutic phlebotomy, making early diagnosis valuable. Recently, a mutation associated with HH has been localized to a cysteine-to-tyrosine mutation at position 282 (C282Y mutation) of the HFE gene (previously called the HLA-H gene), on chromosome 6p, resulting from a guanine-to-adenine transition (1, 2). The C282Y mutation accounts for 83% of the diagnosed HH patients of Northern European ancestry (1, 2). A second mutation, histidine 63 to aspartic acid (H63D), occurs in 17% of the overall population but in 89% of HH patients with C282Y mutations (1). It is unlikely that the H63D mutation is a major contributor to the development of hemochromatosis (3). It should be noted that none of these known HFE mutations are present in all HH cases. Therefore, other mutations, possibly not associated with HFE, contribute to the development of hemochromatosis. Studies by Waheed et al. (4) show a loss of β2-microglobulin binding to the HFE protein having the C282Y mutation. The H63D mutation does not appear to affect β2-microglobulin binding. The disruption of intracellular trafficking of HFE by the C282Y mutation may affect the regulation of intestinal iron absorption through the villial enterocytes (4). However, definitive proof of how the C282Y mutation is involved in HH, is lacking.

The future growth of molecular diagnostics will demand facile, rapid, and inexpensive methods for detecting gene mutations. Most methods for detecting mutations do not meet these criteria, mainly because they require postamplification manipulations. These methods include electrophoresis and solid-phase analysis of amplified products, thereby increasing time and labor and, ultimately, the cost of performing the assay. Moreover, postamplification procedures virtually en-
sure that at some point laboratory contamination of amplified products will occur. Examples of current mutation analysis methods include single-strand conformation polymorphism (SSCP) (5), oligonucleotide ligation assay (OLA) (6), genetic bit analysis (GBA) (7), ligase chain reaction (LCR) (8), and dideoxy sequencing (9). Allele-specific PCR is another popular method; however, this frequently requires extensive optimization and background amplification is often high (10, 12). The least desirable are chemical methods which are time consuming, and frequently employ highly toxic reagents (13, 14).

Recently, peptide nucleic acids (PNAs) have been employed as genotypic probes (15–18). PNAs are polynucleotide mimics having a 2-aminoethylglycine backbone in lieu of the deoxyribose phosphate backbone of DNA (19). PNAs have several advantages over DNA probes. First, PNA–DNA hybrids are more stable than are those for DNA–DNA. Second, sodium concentrations are less influential in the hybridization kinetics of PNA. Third, PNAs are more sensitive to internal base pair mismatches with their DNA complement. A single base-pair mismatch can result in a 10 to 18°C lower melting temperature. Finally, PNAs rarely serve as primers for DNA polymerases (20). Recently, PNA clamping of PCRs has been employed as a means of detecting single base-pair mutations, whereby PNA perfectly complementary to a target region prevents the binding of a PCR primer to the same region (15–18). PNA containing a single base-pair mismatch with respect to the target sequence exhibits minimal inhibition of primer binding.

This method for detecting genetic mutations uses PNA as an allele-specific PCR primer inhibitor, in combination with rapid cycling thermal cyclers that can monitor real-time fluorescence of the double-stranded DNA selective fluorescent dye Sybr Green I (21, 22). Sybr Green I unlike other double-stranded DNA-selective dyes, such as Hoechst 33258 dye, is visible wavelength excitable and provides excellent sensitivity. This mutation analysis method does not require the use of gels or other post-PCR manipulations, thereby reducing assay time and the likelihood for laboratory contamination.

**MATERIALS AND METHODS**

**Materials.** Taq polymerase was from Boehringer-Mannheim. Uracl-DNA glycosidase was from Life Technologies (Gaithersburg, MD). Sybr Green I was from Molecular Probes (Eugene, OR), and the deoxyribonucleotides were from Pharmacia (Piscataway, NJ). All other chemicals were reagent grade.

The LightCycler was from Idaho Technology (Idaho Falls, ID). Genomic DNA from HH patients, HH carriers, and wild type controls were gifts from Roger Wolf of Progenitor Inc. (Palo Alto, CA). The genotypes of the genomic DNA samples were verified by DNA sequencing and were used at Mercator Genetics Inc. as controls in their oligonucleotide ligation assay.

PNA and DNA synthesis. PNA probes were synthesized on a standard nucleotide synthesizer by Ravi Vinayak at Perkin-Elmer ABI. DNA primers and probes were synthesized by Operon Technologies Inc. of Alameda, California.

**Sequences of PNAs**

WT PNA: HAc-CA,CCT,GGC,ACG,TAT-NH$_2$-Gly

MUT PNA: HAc-CA,CCT,GGT,ACG,TAT-NH$_2$-Gly

**Sequences of PCR primers and their complementary oligonucleotides**

WT primer: 5'-GCT,CCA,CCT,GGC,ACG,TAT-3' (9)

MUT primer: 5'-GCT,CCA,CCT,GGT,ACG,TAT-3' (9)

Reverse primer: 5'-CCG,TCT,GGC,ACC,CTA,GTC,ATT-3'

Complement to WT primer: 5'-GAT,ATA,CTG,GCC,AGG,TTG,AGC-3'

Complement to MUT primer: 5'-GAT,ATA,CGT,GCC,ACC,AGG,TTG,AGC-3'

**Melting temperature determinations.** All melting temperature ($T_m$) determinations were performed on a Beckman DU-640 spectrophotometer equipped with a Peltier temperature controller. Temperature ramp rates were 0.5°C per minute, continuously monitoring absorbance at 260 nm. $T_m$ values were calculated from the maximum and minimum values of the first derivative curves of the $A_{260}$-versus-temperature plots using the melting temperature software package for the DU-640 spectrophotometer. DNA probe $T_m$ determinations were performed in PCR buffers containing 3 mM MgCl$_2$. PNA $T_m$ determinations were in 120 mM phosphate, 63 mM NaCl with 0.125% dodecyl dimethylaminopropyl sulfate. PNA and DNA concentrations for the annealing temperature determinations were approximately 1 $\mu$M.

**PNA clamping reaction.** All PCRs contained 50 mM Tris, pH 8.3, 250 $\mu$g/ml BSA, 0.25 $\mu$M PCR primers, 0.2 unit of Taq polymerase, 0.5 units uracl-DNA glycosidase, Sybr Green I at 1:10,000 dilution, 250 $\mu$M deoxyribonucleotides (A, G, C), 500 $\mu$M dU, and 3 mM MgCl$_2$. Experimental variables were 0.5–0.7 ng target DNA (corresponding to 18-fold molar excess over PCR primer).

**LightCycler setup.** Five-microliter aliquots of the above PCRs were loaded into the LightCycler capillary tube along with 3 $\mu$l of light mineral oil. The tubes were sealed in accordance with the manufacturer’s directions and loaded on the LightCycler. Fluorescence was electronically normalized between 40 and 95°C for changes in fluorescence as a function of temperature.
The reaction mixture was subjected to a uracil-DNA glycosidase digestion step of 50°C for 3 min followed by 95°C for 10 min. Thirty-five PCR cycles consisted of a denaturation step to 94°C, ramping at 20°C/s, followed by a 70°C PNA hybridization step for 5 s, ramping at 20°C/s to 63°C, primer annealing step for 15 s, ramping at 3°C/s to 74°C for amplicon extension step for 10 s. A 100-ms fluorescence read was conducted at the end of the 74°C step, using the 510–540 nm emission filter. At the end of 35 cycles an optional melting step was performed to identify amplicons by $T_m$. The melting step consisted of denaturation at 95°C, cooling to 65°C for 20 s, then ramping to 90°C at 0.2°C/s, monitoring fluorescence continuously. Threshold cycle ($C_T$) values are defined as the cycle at which Sybr Green I fluorescence increases significantly above the background as a result of amplicon formation. $C_T$ values were calculated by extrapolation to the x axis of the linear portion of the exponential growth phase. The x intercept, rounded to the nearest cycle number, is the reported threshold cycle.

Gel electrophoresis. The entire 5-μl PCR from the LightCycler and 2 μl of cresol red tracking dye in 20% sucrose were loaded onto a 1.5% agarose gel in TBE buffer. After electrophoresis the gel was stained with ethidium bromide and visualized on a UV transilluminator.

RESULTS

PCRs were conducted as described under Materials and Methods. The PNA clamping scheme is shown in Fig. 1. When PNA contains an internal base-pair mismatch with respect to the target genomic DNA the result is a lowering of the $T_m$ to a point that a perfectly complementary DNA PCR primer will preferentially hybridize to the target DNA. The resulting PCR reaction culminates in an exponential amplification of the target region whereby the amplicon can be detected via fluorescent double-stranded DNA dyes. Conversely, if the DNA primer has a mismatch with respect to the target and the PNA is perfectly complementary, the PNA will preferentially bind to the target DNA, preventing the polymerase from synthesizing nascent DNA. In this scenario, at best, the polymerase can only generate linear amplification of the opposite strand with the reverse primer, and is not likely to be detected by Sybr Green I.

The $T_m$ of the DNA and PNA probes were determined by changes in 260-nm absorbance as a function of temperature. The $T_m$ determinations were performed with complementary oligonucleotides for wild-type or mutant PCR primers as a means of simulating target DNA. For the WT PNA a single base-pair mismatch lowers the $T_m$ by 18°C from 76°C to 58°C. For the MUT pair the $T_m$ decreased by 11°C from 72°C to 61°C (Table 1). The $T_m$ of the WT PCR forward primers with their complement are 65 and 64°C, respectively. Both primers have an annealing temperature of 60°C with a single basepair mismatch (Table 1). In the PCR with homozygous WT genomic DNA, the $T_m$ for the WT PNA is 16°C higher than that for the MUT primer, and the $T_m$ of the WT primer is 4°C higher than that of the

![Fig. 1](image-url) PNA clamping scheme. In scenario (A), the perfectly complementary DNA primer will preferentially bind to the target genomic DNA over the PNA probe, which has an internal mismatch. The result is the formation of amplicon. Scenario (B) is the converse, where the PNA perfectly complementary to the target prevents the mismatched DNA primer from binding. The result is no amplicon formation.
TABLE 1
Melting Temperatures of PNA Probes and Primers

<table>
<thead>
<tr>
<th>Complement to WT primer</th>
<th>Complement to MUT primer</th>
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<tr>
<td>WT PNA</td>
<td>76°C</td>
</tr>
<tr>
<td>MUT PNA</td>
<td>61°C</td>
</tr>
<tr>
<td>WT Primer</td>
<td>65°C</td>
</tr>
<tr>
<td>MUT Primer</td>
<td>60°C</td>
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*M Melting temperatures of PNA–DNA hybrids with and without single base-pair mutations with respect to complementary DNA oligonucleotides. Melting temperatures of PCR primers with their complementary oligonucleotides are also given. See Methods for PNA and DNA sequences.

MUT PNA for complementary DNA. Conversely, in the PCR with the homozygous MUT genomic DNA the T_m for the MUT primer is 6°C higher than that for the WT PNA, and the T_m of the MUT PNA is 12°C higher than that of the WT primer.

For the PNA T_m determinations, it was found that using hybridization buffers containing the zwitterionic detergent dodecyl-dimethylaminopropyl sulfate greatly enhanced the quality of the data. The effect of the detergent is most likely attributable to the lowering of nonspecific binding of PNA to the cuvettes as well as to other PNA molecules.

The PCRs for the genomic DNA analysis were conducted with either the WT PCR primer paired with MUT PNA probe, or with the MUT PCR primer paired with the WT PNA probe. PCRs were run with single patient samples. Each sample was genotypically verified (see Materials and Methods). The DNA primers and PNA probe differ in sequence only at the mutational site and in length. Figure 2 clearly shows that PNA clamping can distinguish between wild-type and mutant homozygotes, in addition to heterozygotes. For the individual with two wild-type alleles the MUT PCR primer/WT PNA pairs showed no reaction whereas the WT PCR primer/MUT PNA pairs generated amplicon (Fig. 2C). The opposite was true with the homozygous HH patient (Fig. 2A). For the heterozygous DNA, both reactions proceed due to one copy each of mutant and wild-type alleles (Fig. 2B). An agarose gel of the LightCycler PCR confirms the LightCycler data where the PNA-suppressed reaction showed no DNA bands by ethidium bromide staining (data not shown). By contrast, the 167-bp band was clearly visible in the reactions where amplicon was detected on the LightCycler (data not shown). There were no primer-dimers visible by ethidium bromide staining for any of the PCRs. The melting temperature of the 167-bp amplicon was determined to be 83°C using the LightCycler melting curve analysis program (data not shown).

Using heterozygous target DNA in a PCR with and without mutant or wild-type PNA, where the PNA probes were at 18-fold excess over primer, delayed the C_T value (threshold cycle) by one cycle (Fig. 3). C_T values were calculated as described under Materials and Methods. Typical C_T values for these PCRs without PNA were approximately 26. On the other hand, in reactions with PNA-directed PCR inhibition the C_T values were increased by an excess of 10 cycles over the noninhibited reaction. This shows that introduction of PNA into the amplification reaction produces minimal nonspecific inhibition of the PCR reaction process.

The data presented are proof of the concept that PNA clamping methods can be readily adapted to PCR instruments, such as the LightCycler, having the capability to monitor real-time fluorescence during the amplification phase.

DISCUSSION

The salient feature of this mutation analysis is the speed at which the assay can be performed, usually in less than 1 h, combined with the lack of post-PCR manipulations of amplicon. The LightCycler reactions are conducted in a sealed glass capillary, thereby minimizing post-PCR release of amplicon. Labeled probes or primers are not needed to perform this assay. Sybr Green I, a double-stranded selective DNA fluorescent probe, serves as a generic probe for quantitation of amplicon. Used at appropriate concentrations, Sybr Green I has minimal effects on the PCR reaction. It should also be noted that rapid cycling PCR usually does not generate primer-dimers, provided that the PCR primers are correctly designed and the annealing temperatures in the PCR reactions are sufficiently high enough to minimize nonspecific hybridization. In the rare instances that primer-dimers are generated, these can be readily distinguished from amplicon on the LightCycler using the melting curve analysis program.

We have shown that this mutation analysis technique is useful for performing rapid genotyping. In these studies we did not incorporate an internal amplification control, such as β-actin PCR primers. In the absence of internal amplification controls there is the possibility that false genotyping will occur if PCR inhibition occurs in one of the two reaction vessels. With the inclusion of internal amplification controls, discrimination of internal control from test amplicons can be performed only with the LightCycler melting curve analysis program, because Sybr Green I will detect all amplification products. The differentiation of multiple amplicons with the LightCycler has been demonstrated by Ririe et al. (23), where amplicons with as little as 2°C differences in T_m can be resolved. Since T_m is based largely on percentage GC content, it is possible to make rational decisions on selection of internal controls for optimal resolution of the gene products on the melting curve analysis program.
Numerous genetic mutation analysis methodologies have been developed. Many use post-PCR analysis of amplicon, thereby greatly increasing assay time and labor and the probability of laboratory contamination of amplicon. Gel-based methods are not likely to gain acceptance where high sample throughput is required. The oligonucleotide ligation assay can be performed in large batches on ELISA plates, but this method is quite labor intensive and labeled nucleotides are still required. Allele-specific PCR is an excellent mutation analysis method. The best performance of allele specific PCR is observed if the 3' end of the PCR primer is the position of the mutation of interest. However, as reported by Kwok et al. (11) some 3' mismatches do show significant PCR amplification. Moreover, having the 3' terminus of the primer as the mutation site generates considerable constraints on PCR primer designs. Conversely, with PNA clamping the interior mu-
tation sites are preferred, enhancing the flexibility of probe and primer designs. Previous PNA clamping studies used molar ratios of 1.5:1-11.4:1 PNA-to-DNA primer to achieve significant inhibition of amplicon formation (15, 16). For our probe and primer sets it was found that 18:1 PNA-to-primer ratios gave optimal results. It is expected that there will be wide variations in optimal PNA-to-primer ratios as a result of variations in length and base composition of primers and PNA probes. We used 21-mer oligonucleotides complementary to the PCR primers as a means of simulating genomic DNA for the purposes of determining melting temperatures of PNA probes and DNA primers. It was found that there was a very broad separation in $T_m$ of WT PNA and MUT primer for complement to WT primer (16°C), and in MUT PNA and WT primer for complement to MUT primer (12°C). Therefore, it is anticipated that there would be minimal amplification formation in these PCRs. In these PCRs, the PNA increases the $C_T$ by 10 or more cycles. On the other hand, the $T_m$ separations between the MUT PNA and WT primer pairs for wild-type complement (4°C) and between the WT PNA and MUT primer for mutant complement (6°C) are very narrow. The possibility that excess PNA could inhibit PCR was investigated. The data shown in Fig. 3 demonstrate that PNA containing a single base-pair mismatch with respect to target DNA elevates the $C_T$ value by one cycle, which will not affect data interpretation. The data presented demonstrate the power and flexibility of PNA clamping for detecting single base-pair mutations. Additionally, PNA clamping methods could also be employed to detect deletion and insertion mutations.

It has also been reported that utilization of a triple strand consisting of parallel and antiparallel PNA and parallel target DNA (PNA$_2$–DNA) creates a better PCR clamping mechanism (15). Considering what is known about the thermodynamics of PNA hybridization and the high stability of the PNA$_2$–DNA triple helix structure this is not surprising. However, the data in this paper, as well as previous literature reports, show that the double-stranded PNA–DNA hybrid is sufficient to arrest the PCR reaction (15–17). Orum et al. (15) demonstrated that PNA clamping can be achieved if the PNA binding overlaps with a primer site (primer exclusion) or when the PNA binding region is immediately adjacent to the primer site. The reason for the latter clamping mechanism is likely due to inhibition of polymerase binding or product extension. No inhibition is observed when the PNA binding site is distant from either primer site (15). Demers et al. (18) showed that PNA binding downstream of the primer sites actually enhances PCR target amplification, in this case, by preventing preferential amplification of small alleles of variable-number tandem repeats.

In conclusion, it appears that PNA can have a variety of uses in a PCR, and what has been done to date is probably a limited example of potential future applications.

**ACKNOWLEDGMENTS**

We thank Ravi Vinayak of Perkin-Elmer for synthesizing the PNA probes. Special thanks go to Roger K. Wolf of Progenitor for providing us with genomic DNA of hemochromatosis patients and hemochromatosis carriers, as well as genomic DNA sequence information.
REFERENCES


