Abstract

**Purpose:** Mutations in mononucleotide repeat sequence (MRS) are good indicators of high-frequency microsatellite instability (MSI-H) cancers, but it has been a challenge to detect such mutations in a large background of wild-type DNA; as in this setting, PCR errors often generate false positive mutant alleles. In this study, we developed a general strategy, referred to as probe clamping primer extension-PCR (PCPE-PCR), to detect MRS alterations in a large background of wild-type DNA.

**Experimental Design:** In PCPE-PCR, genomic DNA is first subjected to PCPE, in which mutant single-strand DNA molecules are preferentially produced. Next, genomic DNA is removed to enrich for the mutant DNA fraction. Thereafter, PCR is carried out using the remaining single-strand DNA molecules as templates. Finally, the PCR products are analyzed to reveal the MSI-H status. In this study, the sensitivity of this new method was first examined by spiking mutant DNA into wild-type DNA at specific ratios followed by studying whether this method is applicable to fecal DNA testing.

**Results:** We showed that PCPE-PCR could detect both mutated BAT26 and transforming growth factor-β-RII (A)10 markers in the presence of 500-fold excess of normal DNA and that as few as three copies of mutated DNA could be detected. In addition, we showed that this technology could detect MSI-H colorectal cancer by fecal DNA analysis.

**Conclusion:** PCPE-PCR is sensitive. In addition, PCPE-PCR is simple and amendable to a cost-effective and high-throughput screening operation. This technology may be applicable to non-invasive screening of MSI-H cancer.

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**High-frequency microsatellite instability (MSI-H) is a form of genetic instability observed in virtually all tumors from patients with hereditary nonpolyposis colorectal cancer and in a subset of various sporadic cancers. Its hallmark is extensive instability in simple repeat nucleotide sequences, including mononucleotide repeat sequences (MRS) and microsatellites (1). MSI-H is most common in colorectal, gastric, and endometrial cancers (2–5). Colorectal cancer is the second deadliest cancer in the United States, and studies identified that about 15% of colorectal cancers are MSI-H, suggesting an annual incidence in the United States of 20,000 to 26,000 MSI-H colorectal cancers each year (3).**

Detection of colorectal cancer by the DNA analysis of stools is a promising screening method (6–10). MRS markers are excellent indicators of MSI-H (11–18). For example, BAT26 alterations were found in >90% of MSI-H colon tumors, and 85% of MSI-H colon cancers harbor a mutation in transforming growth factor-β-RII (A)10 (TGF-β-RII (A)10; refs. 11–14).

Studies show that alterations of these markers are not present in normal tissues, suggesting that they are tumor specific (15–18). Stools contain a small percentage of mutated cells. Thus, molecular assays need to be highly sensitive such that altered DNA can be readily detected in a large background of wild-type DNA. Highly sensitive PCR-based methods are available for the point mutation detection (19–24), but they are not suited for MRS analysis, as PCR itself can produce substantial slippage errors when a locus containing MRS is amplified. In other words, PCR can artificially yield mutant alleles even in the absence of mutant DNA, leading to false positives.

Ahlquist et al. reported on the use of a modified primer extension method for the detection of BAT26 mutations in stool (6, 8). However, primer extension methods may not work well for the detection of mutations in short MRS markers, such as TGF-β-RII (A)10, because polymerase slippage PCR products generated from the wild-type (A)10 allele can substantially overlap with the common (A)9 mutant. Recently, Vogelstein et al. developed
an elegant digital PCR method (25) and successfully employed this approach to detect mutant BAT26 alleles in fecal DNA (9). Digital PCR is sensitive, but the method requires analysis of a large number of subdivided samples each containing a single DNA molecule to screen each patient.

We herein report an alternative detection strategy referred as probe clamping primer extension-PCR (PCPE-PCR) for the detection of MRS alterations in a large background of wild-type DNA. We showed that PCPE-PCR can detect both mutated BAT26 and TGF-RII (A)10 markers in the presence of 500-fold excess of normal DNA, and that as few as three copies of mutant DNA can be detected.

Materials and Methods

Normal DNA was purchased from Sigma (St. Louis, MO). Mutant TGF-RII (A)10 DNA was extracted from cell line of HCT116, and mutant BAT26 DNA was collected from cell line of HEC1A. The low-abundance mutant DNA samples were created by mixing mutant DNA with normal DNA. The abundance and number of mutant DNA in the created samples were estimated based on the number of mutant DNA in the original samples and dilution factors. The stool DNA samples were extracted from stools of MSI-H colorectal cancer patients (9).

In this work, a peptide nucleic acid probe of 5'-GGGTTTTTTTTTCCTTT-TCCT-3' (Applied Biosystems, Foster City, CA) was used for the TGF-RII (A)10 assay, and an oligo probe (5'-GGTGTTTTTTTTTTTTTTTTTTTTTTTTTGAGG-3') was used for the BAT26 assay. The oligo probe was phosphorothioated at the first five positions of the 5' and 3' ends to minimize the probe cleavage by DNA polymerases and was also 3'-end phosphorylated to prevent the probe itself from undergoing primer extension.

For TGF-RII (A)10, PCPE was carried out in 25 μL using 3 μmol/L of the peptide nucleic acid probe, 0.01 μmol/L of the extension primer (5'-biotin-TGCACTCATCAGAGCTACAGG-3'), 0.1 mmol/L of each deoxynucleotide triphosphates, 2 mmol/L of MgCl₂, 1× AmpliTaq Gold PCR buffer, and 0.5 unit of AmpliTaq Gold DNA polymerase.

![Fig. 1. Schematic illustration of the PCPE-PCR principle of detecting mutant DNA (A9) in the presence of a large background of normal DNA (A10).](image-url)
(Applied Biosystems). After denaturation at 95°C for 10 minutes, PCPE was done for 25 to 50 cycles consisting of 30 seconds at 95°C, 120 seconds at 58°C, 60 seconds at 54°C, and 60 seconds at 72°C, with a final extension of 5 minutes at 72°C. After PCPE, the formed single-strand DNA fragments were captured using streptavidin-coated magnetic beads (Dynal Biotech, Lake Success, NY). Twenty-five microliters of extension products were mixed with equal volume of magnetic beads in B&W buffer [10 mmol/L Tris-Cl (pH 7.5), 1 mmol/L EDTA, 2.0 mmol/L NaCl] and incubated at room temperature for 1 to 3 hours. Thereafter, the supernatants were removed followed by washing the beads with 200 μL of 0.1 mol/L NaOH for 5 minutes and twice with water.

A similar procedure was used for BAT26 except for that PCPE was done in 25 to 50 cycles consisting of 30 seconds at 95°C, 120 seconds at 68°C, 60 seconds at 62°C, and 60 seconds at 72°C, with a final extension of 5 minutes at 72°C. The extension primer is 5’-biotin-TGGCAGTTTCATCACTGTCTGC-3’.

The purified beads containing the single-strand DNA fragments served as templates of fluorescence-based PCR. The PCR mixture included 1× PCR buffer, 0.2 mmol/L of each deoxynucleotide triphosphates, 2 mmol/L of MgCl₂, 0.1 mmol/L of the forward and reverse primers, and 0.5 unit of TaqGold DNA polymerase, respectively. After denaturation at 95°C for 10 minutes, PCR (25 μL) was done for 42 cycles consisting of 30 seconds at 95°C, 30 seconds at 54°C, and 30 seconds at 72°C, with a final extension of 5 minutes at 72°C for TGF-β-RII (A)10 and BAT26. The fluorescence-labeled PCR products were size analyzed using a CEPQ8000 sequencer (Beckman Coulter, Fullerton, CA). The forward and reverse primers used were 5’-GAA-GATGCTGCTTCTCCAA and 5’-dye(D4)-ATTGGATATTGCAGCAGTC-3’ for TGF-βRII (A)10, 5’-dye(D4)-ATTGGGATATGCAGCAGTC-3’ and 5’-AACCAATCAACATTTTTAACCC-3’ for BAT26, respectively.

Fecal DNA samples from individuals having MSI-H colon cancers were the kind gift of Dr. Bert Vogelstein (Johns Hopkins University) and corresponded to samples previously employed in studies published by Dr. Vogelstein’s laboratory (9).

Results

A PCPE-PCR assay consists of five steps. First, human DNA is extracted from a clinical sample, such as stools. Second, the DNA samples containing both mutant and wild-type alleles are subjected to PCPE that preferentially produces extension products of mutant DNA. Third, the extension products are extracted, leading to enrichment of mutant DNA. Fourth, PCR is done using the extracted extension products as templates. Finally, PCR products are analyzed using DNA fragment analysis or other methods to reveal the mutations. Figure 1 shows a schematic representation of detecting mutant DNA (A9) in a large background of normal DNA (A)10 by PCPE-PCR.

The second and third steps are the key to PCPE-PCR. As shown in Fig. 1, the PCPE system has an extension primer and a blocking probe, both of which are fully complementary to the same strand of the wild-type sequence. The blocking probe is complementary to a wild-type sequence containing MRS and designed in such a way that probe-DNA duplexes are more stable than extension primer-DNA duplexes when DNA is wild type but are less stable than extension primer-DNA duplexes if DNA is mutated. As a result, this blocking probe can tightly bind to wild-type DNA, preventing read-through by polymerases (elongation arrest); thus, shorter extension products that do not contain the MRS sequence are formed (note that they will not be amplified by the followed PCR as one of the PCR primers will not cover them). In contrast, when DNA is mutated, this blocking probe would no longer bind to the targeted mutation site; thereby, primer elongation is initiated by polymerases, and long extension products containing the altered MRS sequence are formed. In this method, we use solid-phase extraction to extract the extension products. A biotin is incorporated to the extension primer; therefore, the formed extension products are consequently biotinylated as well. The extension products are isolated using a biotin-streptavidin affinity extraction procedure, leading to enrichment of mutant DNA. Thereafter, we use the extracted extension products as PCR templates, and the mutation is revealed by analyzing the PCR products.

In this study, we used the TGF-β-RII (A)10 and BAT26 markers as examples to show the detection of mutations in both short and long MRS markers by this new technology, respectively.

We first used PCPE-PCR to detect mutations of short MRS markers, which contain ≤12 repeats, and that are often altered by only one or two bases. Because of PCR slippage, it can be challenging to detect these mutations in the presence of excessive wild-type alleles. For example, 10% to 30% of the PCR products amplified from the normal TGF-β-RII (A)10 allele can be shortened A9 alleles, making it challenging for conventional methods to detect the legitimate presence of the mutant A9 allele if the abundance of mutant DNA is low. However, the detection of this marker was readily achieved with PCPE-PCR.

Figure 2A shows the PCR product distribution obtained using primer extension but without the blocking probe. The sample contained only wild-type DNA. It was seen that this distribution was identical to that generated from PCR without primer extension, establishing the fact that the sequence of the DNA molecule produced from primer extension truly reflects that of genomic DNA. The A9 peak seen in Fig. 2A was due to PCR slippage, and its intensity was generally <50% of the A10 intensity. Because of this fact, we can use the peak intensity ratio of A9 to A10 to determine whether mutant DNA is present. It was found that a ratio of 0.8 is a good threshold to robustly distinguish the legitimate presence of the A9 allele by polymerases, and long extension products containing the altered MRS sequence are formed. In this method, we use solid-phase extraction to extract the extension products. A biotin is incorporated to the extension primer; therefore, the formed extension products are consequently biotinylated as well. The extension products are isolated using a biotin-streptavidin affinity extraction procedure, leading to enrichment of mutant DNA. Thereafter, we use the extracted extension products as PCR templates, and the mutation is revealed by analyzing the PCR products.

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Fig. 2. TGF-β-RII (A)10 spectra obtained from different conditions. It is noted that (a) PE-PCR denotes the use of primer extension (without the blocking probe) followed by PCR; (b) the percentage indicates the abundance of mutant DNA in the sample; and (c) the peak A9 corresponds to mutant DNA, whereas the peak A10 corresponds to wild-type DNA.
from the artificial production of this allele by PCR slippage. In other words, we called the legitimate presence of the mutant A9 allele in the sample if the ratio is ≥0.8. Otherwise, the absence of mutant DNA would be called. Figure 2B shows the result of detecting 1% (0.5 ng) of mutant DNA in 50 ng of normal DNA without the blocking probe, where no mutant DNA was observed. In contrast, Fig. 2C displays the result obtained from the same sample but with the blocking probe, where the A9 peak became stronger than the A10 peak, indicating the legitimate presence of mutant DNA in the sample assayed. Figure 2D displays the result of detecting 0.1 ng (0.2%) of mutant DNA in 50 ng of normal DNA, where the A9 peak was still stronger, establishing that PCPE-PCR could detect 0.2% of mutant TGF-β-RII (A)10. The amount of human DNA extracted from a clinical specimen may vary from patient to patient; thus, the assay must have a good dynamic range. Figure 2E displays the detection of mutant DNA (0.2%) in the presence of 1 µg of normal DNA, indicating that PCPE-PCR works well in a large dynamic range. It should be noted that the specificity of this assay is good as shown by assaying pure wild-type DNA (Fig. 2F), where the A9 peak was <50% of the A10 peak, correctly indicating the absence of mutant DNA.

We next used PCPE-PCR to detect mutations in large MRS markers. These markers generally contain ≥20 repeats and typically contract by multiple bases. BAT26, one of the most wildly used markers for MSI-H colon cancer, was examined. BAT26 typically contracts by ≥10 bases in MSI-H colorectal cancer but contracts less in adenoma. The data shown here were based on the detection of mutant BAT26 from cell line HEC1A, in which one allele contains about 14As (large deletion), whereas another has 20As (small deletion). The use of this mutant cell line allowed us to evaluate the performance of PCPE-PCR in the detection of both small and large deletions under the same assaying condition.

Figure 3A and B shows the PCR product distribution of both pure normal and mutant BAT26 DNA, respectively. The numbers shown in these spectra specify the size of the corresponding PCR products. The peaks clustering around the positions of 86, 80, and 74 corresponded to the PCR products arising from wild-type BAT26, small deletion in BAT26, and (Fig. 2F), where the A9 peak was <50% of the A10 peak, correctly indicating the absence of mutant DNA.

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large deletion in BAT26, respectively. The formation of multiple peaks resulted from PCR slippage. As seen from Fig. 3A, when there was no mutant DNA, the PCR products of wild-type DNA cluster around peak 86, and the peak intensity of smaller PCR products generally decrease with a decrease in DNA size. For example, peak 80 is weaker than peak 81. However, when mutant DNA was present, the PCR product distribution changed substantially as shown in Fig. 3B. In general, the PCR products of BAT26 with large deletion do not substantially overlap with those of normal DNA, allowing the determination of mutant DNA based on the DNA fragment size. For example, the appearance of peaks around position 74 is indicative of large deletion in BAT26. Although PCR products of BAT26 with small deletion do overlap with those of wild-type DNA, the peak intensity pattern becomes altered due to overlapping. For example, peak 79 is stronger than peak 81 in Fig. 3B. Thus, we were able to determine whether small deletion in BAT26 was legitimately present based on the resulting changes in the peak intensity pattern.

Figure 3C shows the detection of 1% (0.5 ng) of mutant DNA in 50 ng of wild-type DNA without the blocking probe, where no mutant DNA was observed. Figure 3D displays the result obtained from the same sample but with the blocking probe, where new peaks appeared around the peak position of 74, indicating the presence of large deletion in BAT26. In addition, peaks 79 and 80 became stronger than peak 81, suggesting the presence of small deletion in BAT26. Figure 3E displays the result of detecting 0.1 ng of mutant DNA (0.2%) in 50 ng of normal DNA, where the peaks clustering around 74 were seen, revealing the presence of large deletion in BAT26. Moreover, peak 79 was higher than peak 81, indicating the presence of small deletion in BAT26. Figure 3F was obtained using pure wild-type DNA, where neither new peaks nor a change in the peak intensity pattern were observed, indicating the absence of mutant DNA. It is noted that the above experiments were repeated at least twice, and the consistent results were obtained.

Several studies established that the abundance of mutant DNA in stools was generally >0.5% (8–10); thus, an assay with such a sensitivity should be adequate to detect mutant BAT26 from stools (9, 10). Although the BAT26 assay is generally more sensitive to detecting large deletion in BAT26, it is capable of detecting 0.2% of mutant BAT26 that was deleted by six bases, suggesting that the PCPE-PCR-based BAT26 assay could detect both small and large deletion in BAT26 in a sensitive manner. This feature is important to screening as the size of deletion in BAT26 varies among patients.

Because mutant DNA extracted from stools could be scanty (8–10), we also determined the lowest copy of the mutant BAT26 molecules that could be detected in the presence of a 200-fold excessive amount of wild-type DNA. As seen from Fig. 4, three copies of both 12- and 6-base deleted BAT26 were detected in the presence of 600 copies of normal DNA using our BAT26 assay. It was reported that at least 4,000 copies of human DNA could be extracted from 10 g of stools, yielding at least 20 copies of mutant DNA (8–10). Thus, the BAT26 assay is sufficiently sensitive for fecal DNA testing.

We finally tested the BAT26 assay in a set of six fecal DNA samples extracted from stool samples collected from MSI-H colorectal cancer patients to determine whether this assay can detect MSI-H colorectal tumors. Stool DNA was extracted and purified as described previously (9). Because the samples were originally extracted for digital PCR, the total number of human DNA molecules in them is low. Figure 5 shows the result of detecting the BAT26 mutations from one of the stool DNA samples using PCPE-PCR, where the total amount of stool DNA used was about 120 copies. The peaks clustering around the peak position 72 indicated the presence of mutated BAT26, thus revealing that this patient has an MSI-H colorectal tumor. Mutated BAT26 molecules were similarly successfully detected from three of the other assayed fecal DNA samples.

However, two of the six fecal DNA samples from MSI-H patients tested negative for BAT-26 because of failure of observing PCR products. During the course of this work, we found that the assay would become less robust when mutant DNA molecules were less than three copies. This is because robust isolation of extension products became challenging if fewer biotinylated molecules were formed. Because the total number of human DNA in the samples are 120 copies, we suspect in these two negative samples, the copy number of mutant DNA may be <3, which is the minimum level required for PCPE-PCR to detect the mutated BAT26 molecules.
The test is clearly very sensitive; however, the specificity has yet to be completely determined with fecal DNA samples and will need to be further studied in a large number of samples from patients.

**Discussion**

PCPE-PCR offers several unique features. First, PCPE-PCR is sensitive. Second, the same PCPE-PCR assay can be used to detect both large and small deletions. Third, the PCPE-PCR assay uses primer extension as preamplification to increase template copies, allowing the detection of low copies of mutant DNA. This feature is particularly useful in analysis of fecal-derived DNA, where mutant DNA may be scarce. Finally, PCPE-PCR is simple and amendable to a cost-effective and high-throughput operation.

Clearly, the primary application of the PCPE-PCR assay is for noninvasive screening of MSI-H cancers. In the case of colorectal cancer, a combination of sigmoidoscopy and stool analysis has been suggested to be a potentially cost-effective alternative to colonoscopy (9). Sigmoidoscopy inspects the distal colon, whereas PCPE-PCR detects MSI-H tumors, which often occur in the proximal colon. Hence, PCPE-PCR can be a part of a group of methods for screening of colorectal cancers.

It should be noted that the PCPE-PCR strategy reported here is a general strategy and thus can also be used to design assays for the detection of other important MRS markers, including BAT25 and short MRS in BAX (18). If this method is used to detect a marker such as BAT 26, which is polymorphic, it is necessary to test germ line DNA to distinguish the legitimate mutations from polymorphism.

In addition, PCPE-PCR can detect other DNA mutations. In the case of point mutations, our preliminary work indicated that PCPE-PCR could detect p53 mutations in a large background of normal DNA. Hence, PCPE-PCR could potentially serve as a generally applicable method for the detection of various DNA alterations in a large background of normal DNA.

**References**

Detection of Mononucleotide Repeat Sequence Alterations in a Large Background of Normal DNA for Screening High-Frequency Microsatellite Instability Cancers

Xiyuan Sun, Yiding Liu, Jim Lutterbaugh, et al.


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