Application of Peptide Nucleic Acid-based Assays Toward Detection of Somatic Mosaicism

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Peptide nucleic acids (PNAs) are synthetic oligonucleotides with many applications. Compared with DNA, PNAs bind their complementary DNA strand with higher specificity and strength, an attribute that can make it an effective polymerase chain reaction clamp. A growing body of work has demonstrated the utility of PNAs in detecting low levels of mutant DNA, particularly in the detection of circulating mutated tumor cells in the peripheral blood. The PNA-based assay has greater sensitivity than direct sequencing and is significantly more affordable and rapid than next-generation deep sequencing. We have previously demonstrated that PNAs can successfully detect somatic mosaicism in patients with suspected disease phenotypes. In this report, we detail our methodology behind PNA design and application. We describe our protocol for optimizing the PNA for sequencing use and for determining the sensitivity of the PNA-based assay. Lastly, we discuss the potential applications of our assay for future laboratory and clinical purposes and highlight the role of PNAs in the detection of somatic mosaicism.

Introduction

Somatic mosaicism is defined as the presence of two or more genotypes in somatic cells of an individual who was conceived from a single fertilized egg. While uncorrected genetic alterations are thought to occur approximately every two mitotic divisions, the vast majority of these mutations are either deleted through programmed apoptosis or immune surveillance.[1,2] In most cases, mutations that persist beyond this initial checkpoint do not exert any pathologic significance or fail to accumulate to a degree necessary for disease manifestation. However, mutations occurring early in life, especially in the immediate postzygotic period, may provide the genetic predisposing material for later disease development, particularly in regards to cancer.[3] Well-established examples of this phenomenon include McCune–Albright syndrome and tuberous sclerosis, predisposing to development of polyostotic fibrous dysplasias and hamartomas, respectively.[4,5] In addition, diseases classically associated with autosomal inheritance patterns have also been shown to occur sporadically in the context of somatic mosaicism, such as Neurofibromatosis type 1, Neurofibromatosis type 2, and Von-Hippel–Lindau syndrome.[5–7] In recent years, there has been a resurgence of interest in the role of somatic mosaicism in nonsyndromic cases of tumorigenesis and noncancerous human disease.[8–10]

Originally developed by Frederick Sanger and colleagues in 1977, Sanger sequencing has become one of the most widely used methods of DNA sequencing in clinical and basic research. The method utilizes selective incorporation of chain-terminating dideoxynucleotides to sequence predetermined areas of the genome and is both affordable and rapid. However, the sensitivity of this method is limited, particularly

for the detection of somatic mosaicism, with a detection threshold of ~15–20%.[11] In addition, Sanger sequencing of mosaicism present in greater than 60–70% of cells can be mistaken for germline variants.[12] More sensitive sequencing methods such as deep-coverage, "next-generation" sequencing, although increasingly accessible, are still time-consuming and costly.[13]

Peptide nucleic acids (PNAs) are synthetic oligonucleotides, in which the native sugar-phosphate backbone of DNA has been replaced with amino acids.[14] Compared with DNA, PNAs bind their complementary sequences with greater affinity and specificity, due to high resistance to nuclease and protease-mediated degradation. In addition to other diagnostic and therapeutic applications, PNAs represent an affordable and rapid means of testing for somatic mosaicism, by binding to and inhibiting polymerase chain reaction (PCR) amplification of a sequence of interest.

In this report, we argue for the utility of PNAs in the discovery of somatic mosaicism. We describe the design and optimization of PNAs for diagnostic sequencing and discuss recent work from our group, demonstrating the efficacy of PNAs in detecting somatic mosaicism. Lastly, we discuss the advantages and disadvantages of PNA technology and suggest that PNA-based sequencing may be a highly affordable and rapid means of detecting somatic mosaicism.

Results

PNA design

PNAs are synthetic oligonucleotides that share properties of both peptides and nucleic acids. Structurally, the PNA backbone consists of repeating *N*-(2-aminoethyl)-glycine units linked by amide bonds, to which purine and pyrimidine bases are attached. Unlike native DNA, it lacks the pentose sugar moieties or phosphate groups. In addition, unlike DNA, the

PNA backbone is acyclic, achiral, and importantly, neutral, which contributes to higher specificity and stronger binding between complementary PNA/DNA strands than DNA/DNA strands. These properties also cause higher melting temperatures, and thus, PNA hybridization requires higher than normal temperatures during PCR assays. PCR conditions are explained in greater detail below. A deeper discussion of PNA design is outside the scope of this manuscript; however, Nielsen and Egholm,[14,15] who contributed to the original design of the PNA, have explained their work in-depth.

PCR conditions

In our experience, we perform PNA-PCR assays with the following reagents: 200–500 ng genomic DNA, 25 µl Taq 2x Master Mix (New England BioLabs, Ipswich, MA, USA), 0.25 µl each of forward and reverse primers (100 µmol/l), and 0.5 µl PNA (in the optimal concentration; explained in greater detail in the next section) in a final volume of 50 µl. The PCR conditions we use were initially described by Lietman *et al.*[16] and are as follows: denaturation at 94 °C for 15 minutes, followed by 40 cycles of: 94 °C for 30 seconds (denaturation), 68 °C for 60 seconds (PNA hybridization), 55 °C for 30 seconds (primer annealing), and 72 °C for 60 seconds (extension) with a final extension step at 72 °C for 7 minutes.

PNA optimization

The PNA is typically reconstituted into a 100 nmol/l solution for ease of storage. However, in our experience, this results in too high of a concentration of the PNA and completely abrogates amplification of genomic DNA. To determine the upper limit of optimal PNA concentration, 200–500 ng of wild-type genomic DNA is subjected to PCR under PNA-PCR conditions with serially diluted concentrations of the PNA. We typically dilute by 10-fold (1:10, 1:100, 1:1,000, 1:10,000) and later optimize with smaller increments. Subsequently, the PCR products are run under gel electrophoresis, and gels are analyzed under ultraviolet light. The band intensity of the electrophoresed products diminishes as the concentration of the PNA increases, and the PNA concentration at which little to no band is seen is determined to be the upper limit of optimal PNA concentration (**Figure 1a**). We have found that the optimal PNA concentration can vary greatly. For instance, in the aforementioned study examples, the concentrations used for the HIF2A and IDH1 PNAs were 2×10^{-14} mol (1:3,000) and 1×10^{-12} mol (1:1,000), respectively.

PNA application

Once the optimal PNA concentration is determined, the PNA-PCR assay can be applied to the sample DNA of interest, and the reaction products are run under gel electrophoresis. The band is then excised, purified utilizing a standard gel purification kit (MinElute Gel Extraction Kit; Qiagen, Venlo, Netherlands), and sent for routine Sanger sequencing. In the case of somatic mosaicism, a classic double peak or amplified single mutant allele at the nucleotide site may be seen. In instances of absent somatic mosaicism, persistent wild-type sequence is typically observed (**Figure 1b**).

PNA sensitivity

The sensitivity of the PNA is determined by performing the PNA-PCR assay on plasmid mixtures comprised of varying proportions of mutant and wild-type plasmids. The methods are further detailed below.
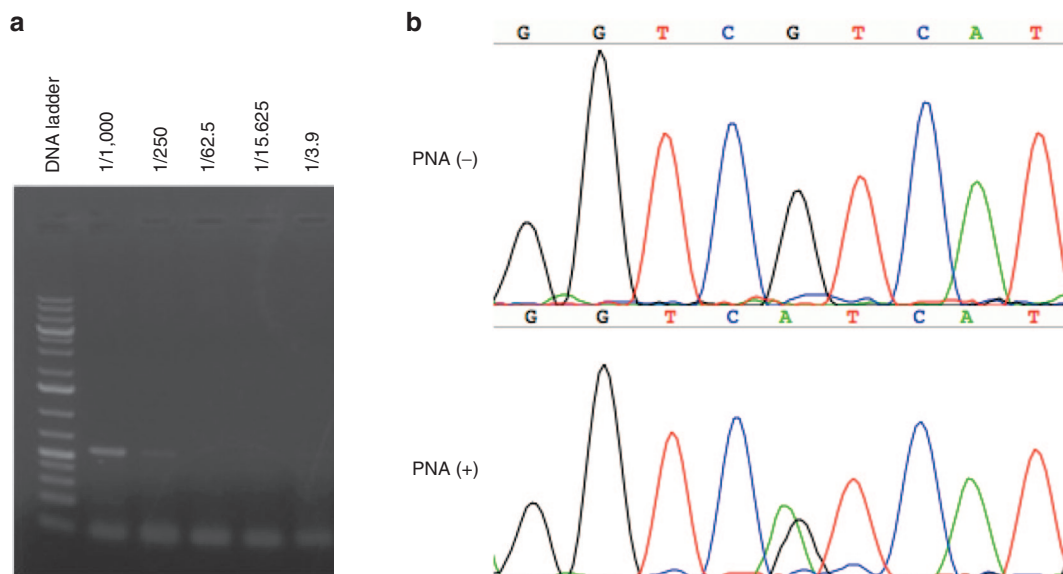

Figure 1 Optimization and application of IDH1 PNA. (**a**) Gel electrophoresis of PCR products derived from IDH1 (codon 132) sequencing of wild-type DNA with serial dilutions of 100 nmol/l IDH1-specific PNA. The far left column is a 1-kb DNA ladder. Subsequently, from left to right are 1,000-fold (1/1,000), 250-fold (1/250), etc. dilutions of 100 nmol/l PNA. The band intensity is significantly reduced from 1/1,000 to 1/250 and is completely absent at 1/62.5, suggesting complete blockage of amplification by the PNA. A dilution of 1/1,000 was chosen as the appropriate dilution for this IDH1-specific PNA. (**b**) Sanger sequencing of IDH1 (R132) from leukocyte DNA is shown with and without PNA. Direct sequencing (top panel) demonstrated wild-type IDH1 R132 status. However, addition of the IDH1-specific PNA revealed an IDH1 (R132H) mutation, suggestive of IDH1 somatic mosaicism. PNA, peptide nucleic acids.

First, mutant and wild-type plasmids are generated through thymine adenine (TA) cloning technology. We utilize heterozygous PCR products from standard PCR of known mutated DNA (*i.e.*, tumor tissue) and clone them into pCR4-TOPO vectors using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). After confirmation of wild-type and mutant DNA sequences of the inserted DNA fragments, the plasmids are amplified with PCR and used for further PCR templates.

Subsequently, wild-type (or mutant) plasmids are diluted in various concentrations, to determine the minimum concentration of plasmids required for the PNA-PCR assay to generate a detectable band on gel electrophoresis. We typically utilize plasmid concentrations ranging from 10 copies/reaction to 10^{10} copies/reaction. In our experience, we have found that ~10^6–10^7 copies/reaction to be the minimum copy number required for gel electrophoresis detection (**Figure 2a**). Next, varying mixtures of mutant and wild-type plasmids are created in which the total number of plasmids per reaction equals the minimum copy number predetermined for gel electrophoresis detection (*i.e.*, 10^7

copies). We typically create mixtures utilizing the following percentages of mutant plasmid DNA in each reaction: 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 2.5, 1.25, 0.6125, and 0.30625%. Next, 1 µl of each plasmid mixture is subjected to the PCR-PNA assay, and all visible bands on gel electrophoresis are then excised, purified, and sent for Sanger sequencing (**Figure 2b**). Utilizing open-access sequencing analysis software (*i.e.*, 4Peaks software; Nucleobytes, Aalsmeer, Netherlands), mutant to wild-type signal ratios measured from each double peak are plotted to generate exponential curves. Lastly, the same experiment is performed with the plasmid mixtures in a standard PCR reaction without the PNA, to generate a control curve. The percentage of mutant plasmid DNA that corresponds to the lowest detectable mutant allele signal on the curve is determined to be the lower limit for sensitivity of the PNA (**Figure 2c**).

Study examples
In this section, we briefly describe two studies recently reported by our group that demonstrate the efficacy of PNAs.
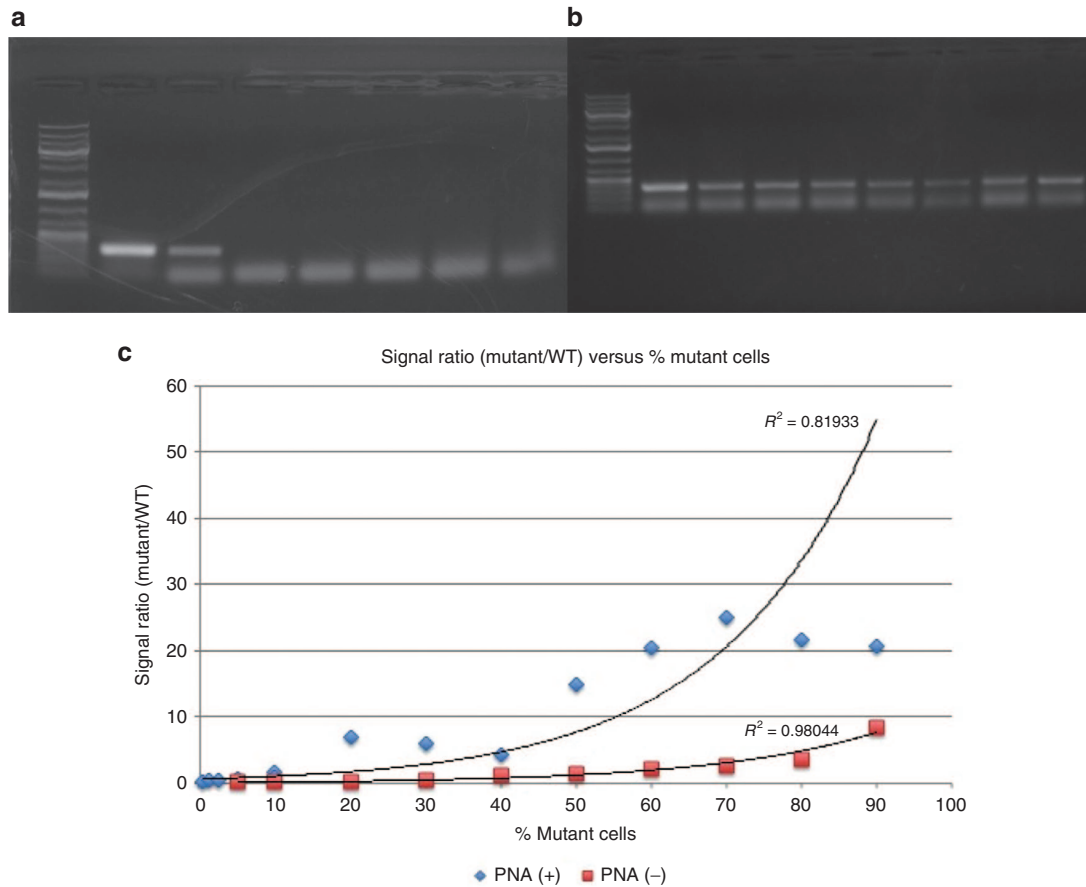

Figure 2 Determination of HIF2A PNA sensitivity. (**a**) Gel electrophoresis of PCR products derived from HIF2A sequencing of HIF2A wild-type plasmids. The far left column is a 1-kb DNA ladder. Subsequently, from left to right are results from 10^7 copies/reaction, 10^6 copies/reaction, 10^5 copies/reaction, down to 10 copies/reaction on the far right. A total copy number of 10^6 per reaction was selected as the proper value from which to perform additional PCR with varying proportional mixtures of wild-type and mutant plasmids. (**b**) Gel electrophoresis of PCR products derived from HIF2A sequencing (with addition of HIF2A-specific PNA) of different proportions of wild-type and mutant plasmids. The far left column is a 1-kb DNA ladder. From left to right are results from plasmid mixtures containing 100% mutant plasmids, 10% mutant plasmids, 5, 2.5, 1.25, 0.6, 0.3, and 0%. Bands were excised and sent for sequencing to determine sensitivity of the HIF2A-specific PNA. (**c**) Results from experiments similar to **b** are graphed, demonstrating the enhanced sensitivity of the IDH1-specific PNA-PCR assay compared with standard sequencing. The IDH1-specific PNA was deemed to be sensitive enough to detect 5% of mutant DNA or 10% of mutated cells. HIF2A, hypoxia-inducible factor 2A; PNA, peptide nucleic acids.

In 2012, we described a novel syndrome of paraganglioma, somatostatinoma, and congenital polycythemia secondary to tumor-specific mutations in hypoxia-inducible factor 2A (HIF2A).[17] At the time, we had hypothesized that the pathophysiology underlying this disorder was somatic mosaicism of HIF2A mutations. However, through standard Sanger sequencing, we were unable to detect HIF2A mutations in germ-line DNA of the two patients we described. Recently, we developed a HIF2A-specific PNA targeting the mutation site present in the patients' tumors. With this PNA, we discovered the presence of the same tumor-specific HIF2A mutation in leukocyte-derived DNA, as well as DNA extracted from hair, nail, and saliva.[18] As such, PNA technology enabled us to validate our initial hypothesis that somatic mosaicism of HIF2A mutations was the genetic mechanism underlying the syndrome of paraganglioma, somatostatinoma, and congenital polycythemia.

In a separate study, we investigated Maffucci syndrome, a disorder characterized by enchondromatosis and benign hemangioma formation. In 2011, Amary and colleagues[19] showed that patients with this condition harbored somatic mosaicism of mutations in isocitrate dehydrogenase 1 (IDH1) or IDH2. However, other tumors have been described in these patients, including those of the central nervous system. Recently, we described a patient with Maffucci syndrome who developed a pituitary adenoma as well as a chondrosarcoma of the skull base.[20] While we were able to detect an IDH1 (R132C) mutation in the chondrosarcoma with standard sequencing techniques, we could not detect the presence of the same IDH1 mutation in tissue from the pituitary adenoma, which was extremely limited and diluted amongst a significant background of nontumoral cells. However, with the aid of an IDH1-specific PNA, we detected the same IDH1 (R132C) mutation in the pituitary adenoma and thus provided genetic evidence that pituitary adenomas could indeed arise within the context of somatic mosaicism of IDH mutations in Maffucci syndrome.

Discussion

In this report, we have demonstrated the efficacy of a PNA-PCR assay to detect low levels of somatic mosaicism in germ-line tissues as well as mutations in cell-heterogeneous samples. Furthermore, we have detailed the methodology utilized by our group to design, optimize, apply, and quantify PNA technology for gene sequencing. Other applications of PNAs are many, including PNA-based probes for fluorescence *in situ* hybridization, PNA-based transcriptomic studies and single-nucleotide polymorphism (SNP) arrays, and therapeutic inhibition of mRNA/micro-RNAs.[21,22] Besides our own reports described above, Lietman and colleagues[16] have utilized a PNA-PCR assay to detect somatic mosaicism of mutant guanine nucleotide binding protein, alpha stimulating (GNAS) alleles circulating in the peripheral blood of patients with McCune–Albright syndrome and fibrous dysplasia. Bushman and colleagues[23] recently applied PNA-based probes targeting amyloid precursor protein to demonstrate somatic mosaicism of amyloid precursor protein in single neurons found in sporadic Alzheimer's disease. Further studies may demonstrate similar applications for PNAs in common diseases of somatic mosaic etiology, including the neurofibromatoses, Von-Hippel–Lindau syndrome, and tuberous sclerosis.

Recently, PNA application in genomic sequencing has extended beyond the diagnostic realm toward prognostic utility. In tumor patients with known mutations, the detection of mutated tumor DNA in the bloodstream may predict long-term prognosis as well as response to therapy. Gonzalez-Cao and colleagues[24] recently showed that a PNA-based assay detecting the BRAF V600E mutation in circulating free DNA had both prognostic and predictive value in assessing response to BRAF inhibitor treatment. Patients with detectable BRAF V600E in their circulation prior to treatment exhibited significantly worsened progression-free and overall survivals, even after targeted BRAF therapy. Likewise, others have shown that PNA-mediated detection of EGFR L858R (exon 21) mutations in the bloodstream of patients with advanced non small-cell lung cancer predicted poorer outcomes, despite treatment with the tyrosine kinase inhibitor, erlotinib.[25]

Like our studies, others have demonstrated the increased sensitivity of a combined PNA-PCR assay, compared with traditional Sanger sequencing.[25–28] In addition, the sensitivity of PNA-based sequencing compares favorably to next-generation deep sequencing, a gold standard for the detection of low levels of mutant DNA, and may generate higher yield use when used together.[29,30] Advantages of the PNA application over deep sequencing include significantly reduced costs, which can equal that of standard Sanger sequencing. In addition, unlike many deep sequencing platforms that require prolonged time for sample analysis, results from PNA-PCR assays can be available within one day, making it suitable for routine clinical use. A major disadvantage of PNA-based sequencing remains the inability to detect novel mutations. PNAs must be designed to target known hotspot mutation sites in the genome, although large-scale PNA-based arrays can be designed to permit one-time testing for multiple genes of interest.[31] Likewise, another limitation of our PNA-PCR assay is the inability to precisely quantify the proportion of mutated cells in a given sample. Although we have previously quantified the degree of somatic mosaicism with TA cloning methods, we have found that a TA cloning-based approach does not work for all samples, particularly those with extremely low levels of mutant DNA.

In conclusion, it is clear from our experience and others that the application of PNA technology toward direct Sanger sequencing yields improved detection of low levels of mutant DNA. In particular, PNAs hold great promise for the detection of somatic mosaicism and understanding disease pathogenesis. Although PNAs remain relatively underutilized, they are a powerful tool and should continue to be incorporated into both basic and clinical research settings.

Materials and Methods

PNAs are commercially available from many different manufacturers. Our group has designed and purchased PNAs from PNA Bio (Thousand Oaks, CA). Other sources that offer custom PNAs include ATDBio (Southampton, UK), Link Technologies (Lanarkshire, Scotland), NeoScientific (Cambridge, MA), and Panagene (Daejeon, Korea).

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