A Highly Sensitive Polymerase Chain Reaction Method Detects Activating Mutations of the GNAS Gene in Peripheral Blood Cells in McCune-Albright Syndrome or Isolated Fibrous Dysplasia

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A Highly Sensitive Polymerase Chain Reaction Method Detects Activating Mutations of the *GNAS* Gene in Peripheral Blood Cells in McCune-Albright Syndrome or Isolated Fibrous Dysplasia

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Background: The somatic nature of mutations in the *GNAS* gene in McCune-Albright syndrome and isolated fibrous dysplasia makes their identification difficult. Conventional methods for the detection of mosaic mutations of *GNAS* have required polymerase chain reaction analysis of genomic DNA from affected tissues or multiple rounds of tandem polymerase chain reaction and endonuclease digestion to enrich for mutant alleles in genomic deoxyribonucleic acid (DNA) from other tissues. Peptide nucleic acid (PNA) primers specifically block synthesis from the nonmutant or wild-type allele. We therefore used PNA-clamping to detect low copy numbers of mutant *GNAS* alleles in DNA from peripheral blood cells from patients with McCune-Albright syndrome and fibrous dysplasia.

Methods: We applied the PNA-clamping method to the analysis of genomic DNA from peripheral blood cells of thirteen patients with McCune-Albright syndrome and three patients with isolated fibrous dysplasia. Polymerase chain reaction was performed in the presence and absence of PNA, and the polymerase chain reaction products were sequenced. In the absence of PNA, a strong 325 base-pair polymerase chain reaction band was generated from all samples; in the presence of PNA, there was an approximately 50% to 90% reduction in the intensity of this polymerase chain reaction product.

Results: In the absence of PNA, direct sequencing of the polymerase chain reaction products demonstrated R201 mutations in *GNAS* alleles of three of the thirteen patients with McCune-Albright syndrome and none of the three patients with fibrous dysplasia. In contrast, in the presence of PNA, R201 mutations were detected in eleven of the thirteen patients with McCune-Albright syndrome and in all three of the patients with fibrous dysplasia. In mixing experiments involving the use of wild-type and mutant DNA samples, we were able to determine the presence of a mutant *GNAS* allele in the equivalent of one cell in 1000 to 5000 cells.

Conclusions: Inclusion of a specific PNA primer in the polymerase chain reaction for *GNAS* exon 8 allows the selective amplification of low numbers of mutant alleles, and it permits detection of activating mutations in genomic DNA from peripheral blood cells in patients with McCune-Albright syndrome and fibrous dysplasia.

Level of Evidence: Diagnostic Level I. See Instructions to Authors for a complete description of levels of evidence.

McCune-Albright syndrome is a sporadic disorder that is characterized by the clinical triad of polyostotic fibrous dysplasia, café au lait skin lesions, and endocrine hyperfunction[1-5]. The prominent features of McCune-Albright syndrome are due to constitutive (i.e., hormone-independent) activation of the adenylyl cyclase signaling pathway in affected tissues and unregulated synthesis of the second messenger cyclic adenosine monophosphate (cAMP). The molecular defect in McCune-Albright syndrome arises from missense mutations in exon 8 of the *GNAS* gene

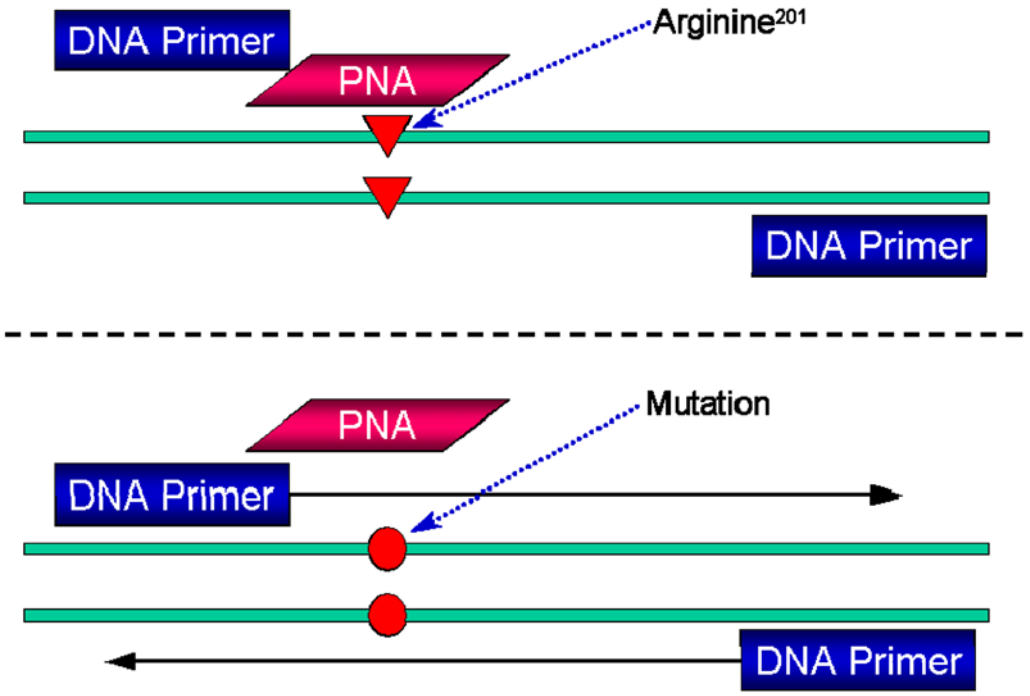

Fig. 1

Illustration depicting the PNA-clamping scheme. In the scenario depicted in the upper panel, the perfectly complementary PNA will preferentially bind to the target wild-type genomic DNA, thus preventing the polymerase chain reaction primer from annealing. This effectively inhibits amplification. Conversely, in the scenario depicted in the lower panel, the PNA probe will not bind to the target DNA because of an internal mismatch in the Arg^{201} codon, thus permitting the polymerase chain reaction primer to anneal. The result is amplification of the mutant *GNAS* amplicon.

that lead to replacement of arginine 201 (Arg^{201} or R201) and conversion to the *gsp* oncogene that encodes the stimulatory guanine nucleotide binding regulatory protein alpha (Gsα) lacking intrinsic GTPase activity[3-5]. Cells containing these activating mutations are present in affected endocrine tissues, skin, and bone from patients with McCune-Albright syndrome as well as in many unaffected tissues, but they are not present in all cells of affected patients, even within affected organs. Mutation-bearing cells are distributed in a mosaic pattern, with the greatest number being present in the most abnormal areas of affected tissues, consistent with the fact that the mutation is not germline but rather is a postzygotic somatic mutation[3-5].

The mosaic pattern of distribution of cells bearing the *GNAS* mutation and the variable number of affected cells in a tissue have hampered ready identification of the gene mutation in peripheral blood cells as mutant *GNAS* alleles may represent only a small proportion of the total number of *GNAS* alleles present in DNA. Detection of mutant *GNAS* genes in DNA samples was previously demonstrated to be enhanced by a protocol in which multiple rounds of nested polymerase chain reaction were performed in conjunction with restriction endonuclease treatment to digest wild-type products, thus enriching the abundance of mutant alleles for selective amplification[6,7].

More recently, Bianco et al.[8] described a protocol involving the use of PNA-clamping[5,9-14] as a means of detecting *GNAS* mutations in DNA samples from fibrous dysplasia lesions (Fig. 1). Karadag et al.[15] further evaluated PNA-clamping with fluorescence resonance energy transfer and noted that this technique was useful for detecting the relative percentage of cells in lesional tissue with an Arg^{201} mutation. PNAs are polynucleotide mimics having a 2-aminoethylglycine backbone in lieu of the deoxyribose phosphate backbone of DNA. PNAs form PNA:DNA hybrids that are more stable than DNA:DNA hybrids are, and PNAs are more sensitive to internal base mismatches with their DNA complement. In this technique, a PNA that is perfectly complementary to the wild-type Arg^{201} nucleotide sequence overlaps the binding site of the forward polymerase chain reaction primer. The PNA prevents the polymerase chain reaction primer from binding to the normal sequence and thereby blocks amplification of the wild-type allele. Because mutant *GNAS* alleles contain a single-base mismatch, the PNA cannot block annealing of the forward polymerase chain reaction primer to mutant *GNAS* alleles, thus enabling selective amplification of the mutant *GNAS* alleles. In the present study, we demonstrate the utility of the PNA-clamping technique for the detection of *GNAS* mutations in genomic DNA isolated from peripheral blood cells of subjects with McCune-Albright syndrome or fibrous dysplasia. While a

negative result on analysis of peripheral blood (observed in two of the patients in the present study) does not exclude the diagnosis of McCune-Albright syndrome or fibrous dysplasia, the ability to diagnose McCune-Albright syndrome or fibrous dysplasia on the basis of blood samples could prevent many diagnostic biopsies and thereby avoid complications such as fracture, infection, or anesthetic complications.

Materials and Methods

The present study was approved by the internal review board at our institution. Consent was obtained from all patients (or from their family members) prior to inclusion in the study. Peripheral blood samples were obtained from individuals with either McCune-Albright syndrome or fibrous dysplasia during the course of routine care or research evaluation (see Appendix). Whole blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA), and leukocytes were isolated with the Histopaque kit (Sigma, St. Louis, Missouri) as recommended on the package insert. DNA was then obtained by means of phenol chloroform extraction.

A PNA probe was synthesized on a PerSeptive Biosystems Expedite instrument (PerSeptive Biosystems, Framingham, Massachusetts) by Dr. Nga Nguyen at the National Institutes of Health (Center for Biologics Evaluation and Research, Facility for Biotechnology Research, Peptide Laboratory) and was purified by means of reverse-phase high performance liquid chromatography. The sequences of the PNA (base pairs 642 to 653 for codons 199 to 202) and primers used to amplify a portion of exon 8 of *GNAS* were as follows:

PNA: Gly-NH_2-CGCTGCCGTGTC-HAc
Forward primer: 5′-GTTTCAGGACCTGCTTCGC-3′
Reverse primer: 5′-GCAAAGCCAAGAGCGTGAG-3′

Each polymerase chain reaction contained 200 to 500 ng of target genomic DNA, 2.5 U of AmpliTaq Gold polymerase (PerkinElmer, Boston, Massachusetts), 1 μg each of forward and reverse polymerase chain reaction primer, and 2 μg of the PNA in a 100-μl final volume. After an initial denaturation at 94°C for fifteen minutes to activate the polymerase, forty polymerase chain reaction cycles followed, consisting of a denaturation step to 94°C for thirty seconds, a PNA hybridization step at 68°C for sixty seconds, a primer annealing step at 55°C for thirty seconds, and an extension step at 72°C for sixty seconds. A final extension step was performed at 72°C for seven minutes[8].

At the conclusion of the polymerase chain reaction, the sample was electrophoresed through a 5% polyacrylamide gel in TBE buffer, stained with ethidium bromide, and visualized on an ultraviolet transilluminator. Bands corresponding to an expected 325-base pair (bp) product were isolated from the polyacrylamide gel and were directly sequenced by polymerase chain reaction with use of the forward primer and the Thermo Sequenase radiolabeled terminator cycle sequencing kit according to the manufacturer's instructions (Amersham Biosciences, Piscataway, New Jersey). For mixing experiments, the DNA sample from the patients with McCune-Albright syndrome contained equal amounts of wild-type and mutant

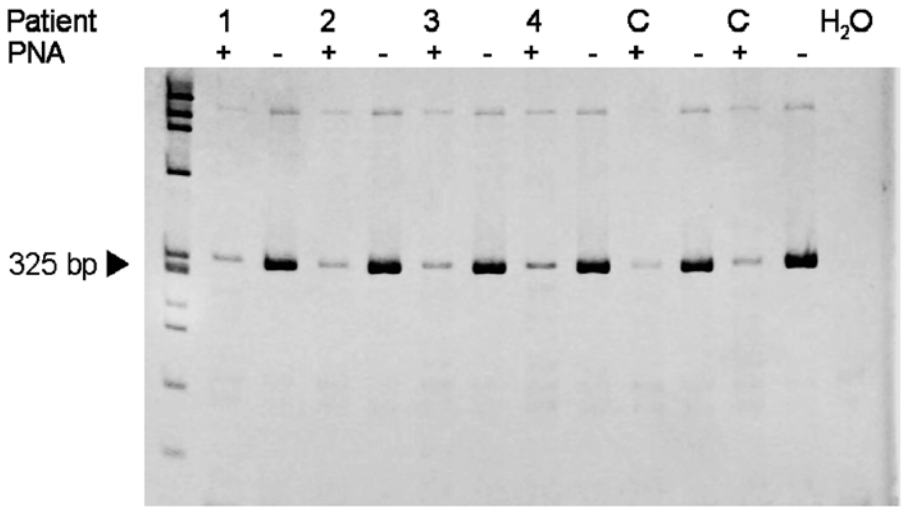

Fig. 2

PNA inhibited polymerase chain reaction of the wild-type *GNAS* gene. Polymerase chain reaction was performed in the presence (+) or absence (–) of PNA with use of genomic DNA from subjects with McCune-Albright syndrome (lanes labeled 1 through 4) and two normal control individuals (lanes labeled C). A band of predicted size (325 base pairs) was present when polymerase chain reaction was performed in the absence of PNA; the intensity of this band was reduced by the inclusion of the PNA and was nearly absent in the DNA samples from normal control subjects. These samples were loaded on a 5% polyacrylamide gel; an inverse image is presented. The water lane represents a control with no DNA; there is no amplicon in this water lane, indicating no contamination.

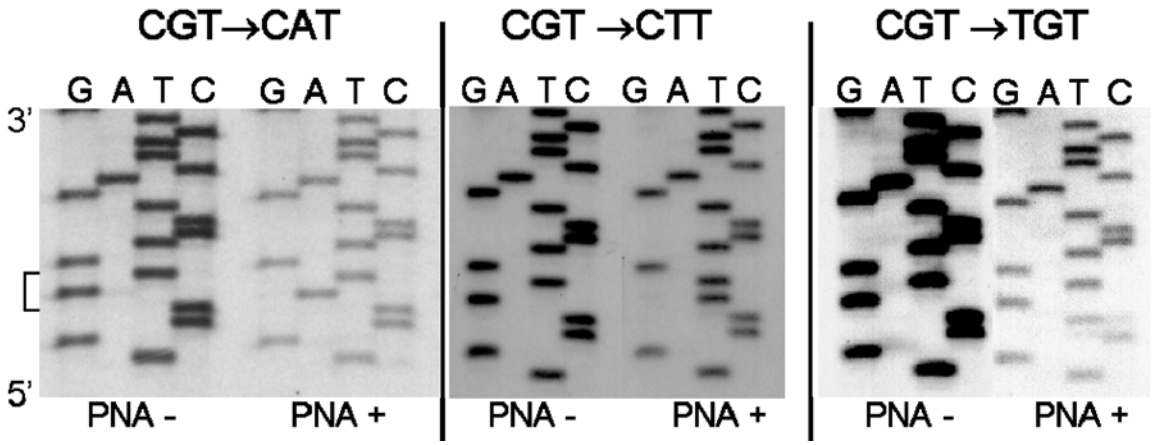

Fig. 3

Analysis of *GNAS* mutations in DNA isolated from peripheral blood leukocytes. Nucleotide sequence analysis of the region encompassing the arginine 201 (Arg^{201}) codon is presented for three subjects with McCune-Albright syndrome. Polymerase chain reaction performed in the absence of PNA revealed only alleles containing wild-type sequence (CGT). In contrast, when polymerase chain reaction was performed in the presence of PNA, only alleles containing *GNAS* mutations were amplified. Three different mutations were detected with equivalent sensitivity: CGT→CAT/arginine→histidine (arg→his), CGT→CTT/arginine→leucine (arg→leu), and CGT→TGT/arginine→cysteine (arg→cys).

GNAS alleles (i.e., 50% mutant alleles) and was mixed with increasing amounts of normal DNA to obtain the indicated percentages of mutant alleles.

Results

All of the patients with McCune-Albright syndrome had café au lait skin lesions, fibrous dysplasia bone lesions, and endocrinopathy and therefore met the clinical diagnostic criteria for McCune-Albright syndrome[1-5]. All of the patients with fibrous dysplasia had only fibrous dysplasia bone lesions and therefore met the clinical diagnostic criteria for fibrous dysplasia.

In the absence of PNA, a strong 325-bp polymerase chain reaction band was generated from all samples and direct sequencing of the polymerase chain reaction products demonstrated R201 mutations in only three of the thirteen patients with McCune-Albright syndrome and in none of the three patients with fibrous dysplasia. When polymerase chain reaction was performed in the presence of PNA, there was an approximately 50% to 90% reduction in the intensity of this polymerase chain reaction product (Fig. 2).

In the presence of PNA, direct sequence analysis revealed the R201 mutation in eleven of the thirteen patients with McCune-Albright syndrome (four patients with R201H, six patients with R201C, and one patient with R201L) (Fig. 3) and in all three patients with fibrous dysplasia (all with R201C). In experiments involving the use of wild-type and mutant DNA samples mixed together, we were able to determine the presence of a mutant *GNAS* allele in the equivalent of one cell with the mutation among 1000 to 2000 normal cells (representing a rate of detection of 0.1% to 0.05%) (Fig. 4). DNA samples from normal subjects served as a negative control and showed wild-type *GNAS* sequence in the absence or presence of PNA and no mutation in the presence of PNA (data not shown).

Discussion

The mosaic pattern of distribution of cells bearing the *GNAS* mutation, and the variable number of affected cells in a tissue, has complicated the identification of mutant *GNAS* alleles, which may represent only a small proportion of the *GNAS* alleles present in DNA isolated from tissue or peripheral blood. Detection of mutant *GNAS* genes in DNA samples had been previously enhanced by a protocol in which multiple rounds of nested polymerase chain reaction were performed in conjunction with restriction endonuclease treatment to digest wild-type products, thus enriching the abundance of mutant alleles for selective amplification[6,7]. However, this technique is not applicable for all mutation sites and has been shown to have variable sensitivity[16]. Bianco et al.[8] and Karadag et al.[15] previously applied the PNA-clamping technique to analyze lesional tissue from patients with McCune-Albright syndrome, which suggested to us that this approach might be useful for analyzing peripheral blood samples from patients with McCune-Albright syndrome or isolated fibrous dysplasia. While the presence of café au lait and endocrine abnormalities in association with McCune-Albright syndrome and fibrous dysplasia suggests that there might be cells in the blood with *GNAS* mutations, and while several investigators have shown that humoral factors are involved in hypophosphatemic rickets accompanying McCune-Albright syndrome, no one to our knowledge has been able to consistently find the mutation in peripheral blood samples, and, in general, the diagnosis is still made clinically[2,17,18]. In the present study, we found that PNA-clamping provided an enhancement over standard polymerase chain reaction as well as a simple alternative to nested polymerase chain reaction

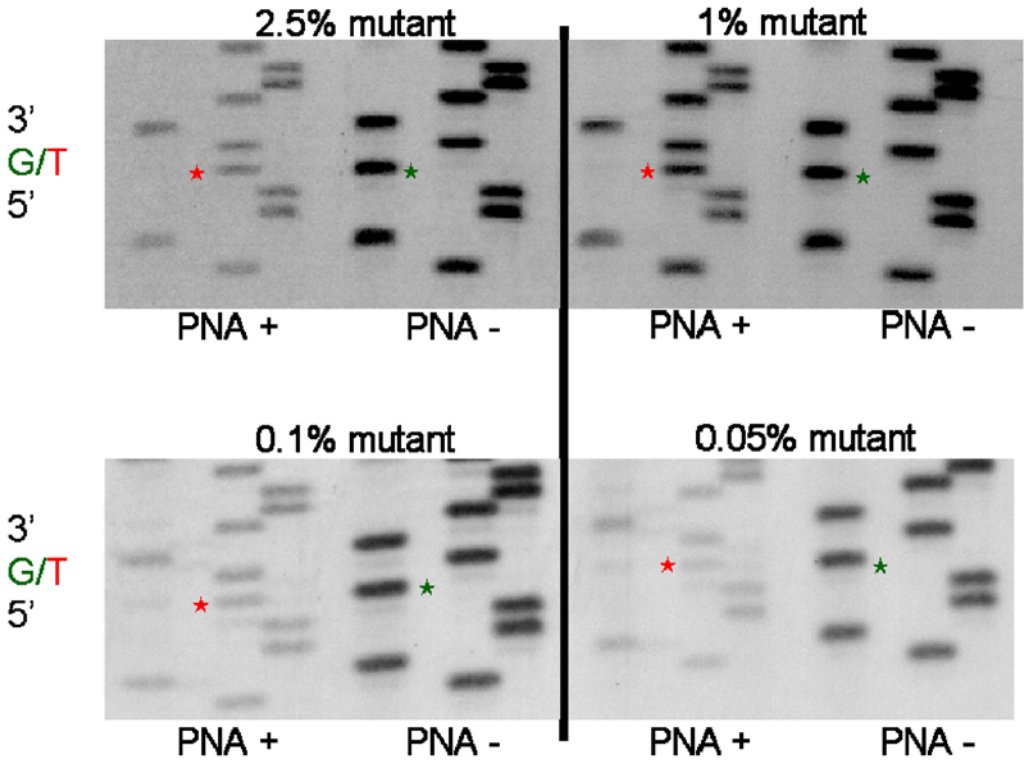

Fig. 4

Sensitivity of the PNA-clamping method. Nucleotide sequence analysis of the region encompassing the Arg^{201} codon is presented after polymerase chain reaction of DNA containing varying amounts of genomic DNA from a subject with McCune-Albright syndrome and a normal subject. The DNA sample from the patient with McCune-Albright syndrome contained equal amounts of wild-type and mutant *GNAS* alleles (i.e., 50% mutant alleles) and was mixed with increasing amounts of normal DNA to obtain the indicated percentages of mutant alleles. When polymerase chain reaction was performed in the absence of PNA, sequence analysis was unable to detect the *GNAS* mutation unless mutant alleles constituted >2.5% of the target genomic DNA. In contrast, when polymerase chain reaction was performed in the presence of PNA, the *GNAS* mutation was easily detected even in DNA samples in which the mutant DNA constituted only 0.1% of the total.

schemes in the detection of *GNAS* mutations and that it detected *GNAS* mutations in genomic DNA isolated from peripheral blood leukocytes in eleven of thirteen patients with McCune-Albright syndrome and three of three patients with fibrous dysplasia. The ability to detect *GNAS* mutations in DNA isolated from peripheral blood of patients with McCune-Albright syndrome implies that the leukocytes contain mutant *GNAS* alleles and/or that cells from affected tissues are also present in the circulation.

Similarly, the presence of cells bearing the *GNAS*-activating mutation in the circulation of patients with fibrous dysplasia suggests either that the somatic mosaicism is more widespread than clinically suspected or that cells from fibrous dysplasia lesions are shed into the circulation. Additional studies will be needed to distinguish between these two alternatives, and some patients may not have detectable mutations in the blood if their involvement does not extend to the peripheral blood. In conclusion, our study adds support to the growing enthusiasm for and utility of PNA-clamping in molecular diagnostics. The ability to identify somatic mutations in cells that represent only a small percentage of the total cell mass may allow this technique to be adapted for the analysis of blood or tissues in patients with malignant disease in order to increase the sensitivity of cancer-staging and therefore improve decisions about the need for and efficacy of systemic treatment.

Appendix

eA A table presenting clinical details of all study subjects is available with the electronic versions of this article, on our web site at www.jbjs.org (go to the article citation and click on "Supplementary Material") and on our quarterly CD-ROM (call our subscription department, at 781-449-9780, to order the CD-ROM).

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