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PREVALENCE OF THREE MUTATIONS IN THE $G_s\alpha$ GENE AMONG 24 FAMILIES WITH PSEUDOHYPOPARATHYROIDISM TYPE Ia[1]

Cal K. Lin, Michael J. Hakakha, Jon M. Nakamoto, Anna T. Englund, Arnold S. Brickman,* Marilyn L. Scott, and Cornelis Van Dop

Division of Pediatric Endocrinology and Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, CA 90024

*Section of Mineral Metabolism, Sepulveda Veterans Administration Medical Center, Sepulveda, CA 91343

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Pseudohypoparathyroidism type Ia (PHP-Ia), an inherited multi-hormone resistance syndrome, is associated with deficient cellular activity of the α-subunit of the guanine nucleotide-binding protein ($G_s\alpha$) that stimulates adenylyl cyclase. We determined prevalence of three recently described mutations in exons 1 and 10 of the $G_s\alpha$ gene among 24 unrelated patients with PHP-Ia. Restriction analysis was used to detect two mutations that produce unique RFLPs, and allele-specific oligonucleotide hybridization was used to detect the other mutation. As none of these mutations were not found, genomic DNA was analyzed with denaturing gradient gel electrophoresis to screen for other mutations in exon 10. Mutations of the initiation codon and exon 10 in the $G_s\alpha$ gene thus rarely ($\leq$4% each) cause PHP-Ia and the $G_s\alpha$ gene mutations causing PHP-Ia are heterogeneous and unique to each pedigree.

Pseudohypoparathyroidism type Ia (PHP-Ia) is a rare genetic disorder of renal resistance to parathyroid hormone (PTH) with hypocalcemia, hyperphosphatemia, and deficient urinary excretion of cyclic AMP after administration of PTH. The disease results from a generalized

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Abbreviations used: AHO, Albright hereditary osteodystrophy; $G_s\alpha$, guanine nucleotide-binding coupling protein that stimulates adenylyl cyclase; nt, nucleotide; PHP-Ia, pseudohypoparathyroidism type Ia.

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50% reduction in cellular activity of the α-subunit of the guanine nucleotide binding protein ($G_s\alpha$) that stimulates adenylyl cyclase (for review see ref. 1). Recently, three different mutations of the $G_s\alpha$ gene were described in three families with AHO and PHP-Ia (2,3) in which the $G_s\alpha$ gene mutation co-segregated in a dominant inheritance pattern with the AHO phenotype. We undertook this study to determine the prevalence of these three specific mutations among patients with PHP-Ia.

MATERIALS AND METHODS

Patients: Study subjects were 24 unrelated patients with PHP-Ia who manifested either renal resistance to PTH or elevated serum concentrations of thyrotropin without evidence of Hashimoto's thyroiditis. Most had undergone PTH infusions tests that demonstrated deficient rises in urinary phosphate and cyclic AMP and all had both ~50% reduction in erythrocyte $G_s\alpha$ activity and AHO evidenced by short stature or brachymetacarpia. The study protocol was reviewed and approved by the Human Subjects Protection Committee at UCLA, and informed consent was obtained from the subjects prior to this study. Erythrocyte $G_s\alpha$ activity was measured as described (4).

Southern restriction analysis of genomic DNA with NcoI: Genomic DNA was isolated, and restriction blots were prepared on GeneScreen Plus (DuPont, DE) membranes and probed with ^{32}P-labeled cDNA probes (5). Hybridization probe was a 440-bp fragment of the human $G_s\alpha$ cDNA from the *NcoI* site at the initiation codon to a downstream *SphI* site and was ^{32}P-radiolabeled (S.A. ~10^8 cpm/μg) using random priming (6).

Polymerase chain reaction (PCR): Two oligonucleotides with sequences complementary to the anti-sense strand in the intron upstream of exon 9 and to the sense strand in the intron located downstream of exon 10 were synthesized (Table 1). One was synthesized with a 36-nt addition at the 5' end consisting of random sequence guanine and cytidine deoxynucleotides. Genomic DNA from patients and control subjects was sheared before use. Each PCR reaction (50 μl) contained 10 mM Tris-Cl pH 8.3, 1.5 mM $MgCl_2$, 0.001% gelatin, 50 μM each dNTP, 0.31 μM each primer, and 1-2 μg sheared genomic DNA from the study subject. The reaction was capped with 50 μl mineral oil, heated to 95°C for 10 min, and then cooled to 80°C before addition of 1.25 U *Taq* DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT). The reaction was then cycled 35 times between 94°C for 1 min and 65°C for 2 min before incubation for 5 min at 72°C. Agarose gel electrophoresis was used to confirm amplification of the appropriate DNA fragment.

Allele-specific oligonucleotide hybridization: Duplicate dot blots were prepared on GeneScreen Plus from PCR-amplified DNA after serial dilutions with 0.2-50 ng DNA per dot. PCR-amplified DNA was denatured with 0.4 M NaOH/25 mM EDTA and boiled for 2 min, before rapid cooling on ice. The oligonucleotides used for allele-specific hybridization (Table 1) were end-labeled to the same specific activity with [γ-^{32}P]ATP and polynucleotide kinase. After prehybridization for 24 h at 37°C in 5x SSPE/5x Denhardt's solution/0.5% SDS, duplicate blots were hybridized in parallel overnight with the ^{32}P-labeled oligonucleotides containing either wild-type or mutant sequence. Blots were then washed 10 min 3x with 6x SSC at RT before 3 M tetramethylammonium chloride/2 mM EDTA/0.1% SDS/50mM Tris-Cl pH 8.0 for 20 min at RT and then 2x in this solution at 54°-56°C. The blots then exposed Kodak XAR-5 film with intensifying screens at -75°C. Similar results were obtained using dot blots and radiolabeled probes that had been independently prepared.

Restriction analysis with AluI: The DNA fragment containing exons 9-10 of $G_s\alpha$ was amplified from genomic DNA of all 24 study subjects and 17 control subjects using PCR. PCR-amplified DNA was digested with *Alu*I, end-labeled using [γ-^{32}P]ATP and polynucleotide kinase, and size-fractionation on a polyacrylamide-TBE gel and subsequent autoradiography (Fig. 3).

GC-Clamped denaturing gradient gel electrophoresis: The method described by Myers *et al.* (7) was used to screen for mutations in PCR-amplified DNA from exons 9-10 of the $G_s\alpha$ gene. PCR with GC-tailed oligonucleotide primers was used to amplify exons 9-10 of the $G_s\alpha$ gene from genomic DNA of the 24 patients and 20 normal control subjects. PCR-amplified DNA ($\sim$2 μg) was fractionated at 60°C on 7% polyacrylamide gels with linear vertical denaturant gradients of urea-formamide ranging from 10 to 50% and 40% to 80% (100% denaturant = 40% formamide/7 M urea) in 0.5x TBE. Gels were 13 cm long (Green Mountain Lab Supply, MA) and were electrophoresed for 6 h at 150 V. DNA was then visualized with UV illumination after staining with ethidium bromide.

Chemicals and reagents: Chemicals and reagents were purchased commercially and were the highest quality available. Oligonucleotides (Table 1) were synthesized on a Cyclone Nucleotide Synthesizer (Milligen) using phosphoramidite derivatives of nucleotides.

RESULTS

Mutations of the initiation codon: The recognition sequence for *Nco*I, CCATGG, occurs in exon 1 of the $G_s\alpha$ gene and includes the initiation codon of $G_s\alpha$ (8). From restriction analysis (2), *Nco*I restriction sites are located $\sim$1-kb upstream of the $G_s\alpha$ initiation codon and $\sim$0.5-kb downstream in the intron between exons 1 and 2. As the cDNA probe used in our studies extends 440-bp 3' from the initiation codon, only one of these two fragments, the 0.5-kb fragment, derived from wild-type sequence exon 1 will be demonstrable. Although all *Nco*I restriction blots demonstrated the 0.5-kb fragment (Fig. 1), none of the DNA from 24 study subjects or 17 control subjects contained the larger 1.5-kb *Nco*I RFLP described by Patten *et al.* (2). As the *Nco*I site includes the entire initiation codon, any nucleotide changes within the initiation codon also eliminate this *Nco*I site to yield the 1.5-kb RFLP. In addition to the 0.5-kb fragment from exon 1, 3.3-kb and 3.9-kb fragments (Fig. 1) hybridized with the cDNA probe in DNA from all patients and control subjects. These DNA fragments resulted from hybridization of the cDNA probe to *Nco*I restriction fragments containing exons 2 to 5 of the $G_s\alpha$ gene. Absence of the 1.5-kb *Nco*I restriction fragment among the patients with PHP-Ia indicates that none had either the mutation described by Patten *et al.* (2) or other mutations that alter the initiation codon in the $G_s\alpha$ gene.

Detection of mutations in exon 10 of $G_s\alpha$: The mutation causing the exon 10 frame-shift was not detected among the PHP-Ia patients or normal subjects using allele-specific oligonucleotide hybridization to PCR-amplified DNA from their exons 9-10 of the $G_s\alpha$ gene (Fig. 2).

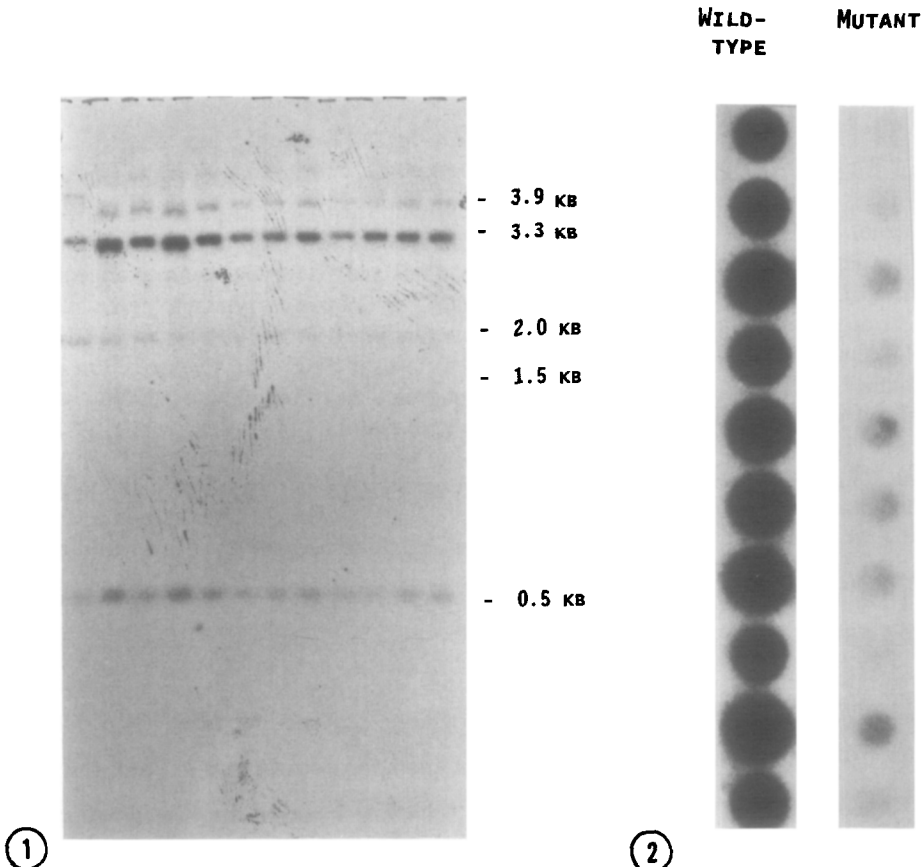

Figure 1. Autoradiograph of *Nco*I restriction blot prepared from genomic DNA of subjects with AHO and probed with radiolabeled $G_s\alpha$ cDNA. Genomic DNA (7 μg) from each study subject was completely digested with *Nco*I and size-fractionated on a 2% agarose-TBE gel. After transfer to support membrane by capillary blotting, the blot was probed with a ^{32}P-radiolabeled 440-bp fragment containing codons 1-147 of human $G_s\alpha$ cDNA. On the right side are indicated hybridization fragment sizes and location for a 1.5-kb RFLP resulting from any mutation within the initiation codon of the $G_s\alpha$ gene.

Figure 2. Allele-specific oligonucleotide hybridization. Autoradiograph of representative dot blot hybridized with allele-specific oligonucleotides (see Table 1) to 5 ng PCR-amplified DNA from $G_s\alpha$ exons 9-10 in PHP-Ia patients. Hybridization with oligonucleotides specific for sequence at the exon 10 reading frame-shift mutation, with wild-type sequence (left) and mutant sequence (right).

The mutation at the splice acceptor site on exon 10 (3) introduces an *Alu*I restriction site; therefore we screened for this mutation using *Alu*I restriction analysis of PCR-amplified DNA from exons 9 and 10. Using the oligomers defined in Table 1 yields a 370-bp fragment, as predicted from the genomic DNA sequence (8). This amplified DNA fragment from the wild-type gene contains one *Alu*I site that cuts the fragment into 210-bp and 160-bp fragments. The splice-junction mutation (3) introduces a second *Alu*I site into this fragment so that the 160-bp fragment homologous to the wild-type sequence yields a 84-bp and 76-bp

TABLE 1. SYNTHETIC OLIGONUCLEOTIDES

$G_s\alpha$ gene region	Oligonucleotide sequence
A. Allele-specific oligonucleotides	
Exon 10 frame-shift	
Wild-type	5'-dTCTGAACCTCTTCAAGAGC-3'
Mutant	5'-dTCTGAACTCTTCAAGAGCA-3'
B. Primers for PCR amplification of exons 9-10 from genomic DNA	
Sense	5'-dGCCGCCCGCCGCCCGCCGCGCCGCCCGCCGCCCGCC-CCAGTCCCTCTGGAATAACCAGCTGTC-3'
Antisense	5'-dCGGGGTTCTTCTCTATAAACAGTGCAGAC-3'

fragments. Absence of these ~80-bp fragments from DNA of all the patients and control samples did not result from incomplete *Alu*I digestion as incomplete digestion of the mutant DNA fragment would yield some of the shorter fragment in addition to the prominent 160-bp fragment. Absence of radiolabeled *Alu*I fragments with length ~80-bp on autoradiographs thus demonstrated that none of the 24 PHP-Ia patients or the 17 control subjects had this mutation (Fig. 3).

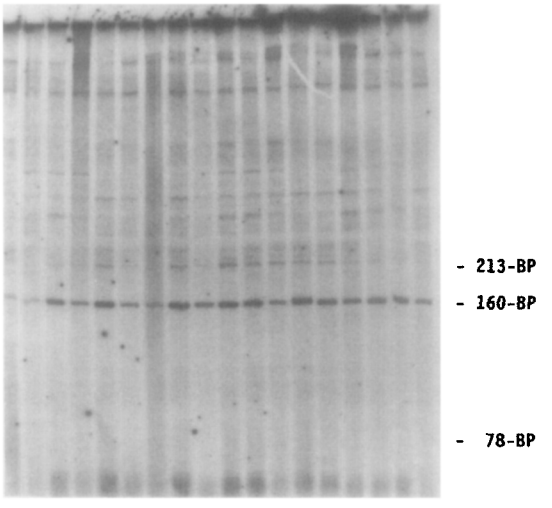

Figure 3. Autoradiograph of *Alu*I restriction analysis of DNA from exons 9-10 in the $G_s\alpha$ gene. A DNA fragment containing exons 9 and 10 of the $G_s\alpha$ gene was amplified from genomic DNA of PHP-Ia patients using PCR and digested with *Alu*I. After radiolabeling the fragments with ^{32}P, the DNA was size-fractionated electrophoresis in an agarose-TBE gel and autoradiography was completed. Fragment lengths are indicated, including the position for ~80-bp fragments expected to result if the G to C transversion in the exon 10 splice acceptor site was present.

Finally, denaturing gradient gel electrophoresis of GC-clamped PCR-amplified DNA fragments failed to detect any sequence polymorphisms in the exons 9 and 10 of the $G_s\alpha$ gene in DNA from the 24 PHP-Ia patients and 20 unrelated control subjects (data not shown). The absence of DNA polymorphisms during DGGE analysis of genomic DNA verified the results obtained for exon 10 using allele-specific oligonucleotide hybridization.

DISCUSSION

The pedigrees that we studied had varied ethnic backgrounds and included one African-American family and two Hispanic families. However, neither Patten *et al.* (2) nor Weinstein *et al.* (3) reported the ethnic origin of their patients; therefore, we cannot exclude the possibility that a unique ethnic background of their patients accounts for the apparent low overall prevalence of their mutations among our patients with PHP-Ia.

In their original report of the frame shift and splice acceptor site mutations in exon 10, Weinstein *et al.* (3) did not detect these same mutations of exon 10 in six other families with PHP-Ia that they screened. Combining their prevalence data with our own, the two mutations in exon 10 thus each occurred in only one of 30 families with a resultant estimated prevalence of $<4\%$ among patients with PHP-Ia. Patten *et al.* (2) did not report screening any other PHP-Ia families for the mutation of the initiation codon; thus similar combination of their data with ours yielded a similar low prevalence (1/25) for this mutation among pedigrees with PHP-Ia. The total number of families screened for each mutation remains small, but screening more families may be difficult except via multi-center screening programs, considering the rarity of PHP-Ia. Although our families with PHP-Ia did not have any of these three mutations, they presumably bear other mutations that cause reduced cellular $G_s\alpha$ activity.

In fact, we have discovered mutations in other exons of the $G_s\alpha$ gene among our PHP-Ia pedigrees (9). Like the mutations described by Patten *et al.* (2) and Weinstein *et al.* (3), those mutations occurred in single PHP-Ia families, except one mutation which was found in two unrelated patients (10). Our results indicate that mutations of the $G_s\alpha$ gene that cause PHP-Ia are heterogenous. Thus DNA-based screening for these specific mutations in the $G_s\alpha$ gene among families with PHP-Ia is not useful. However, measurement of $G_s\alpha$ activity in erythrocytes of subjects with PHP can be useful for identifying patients with PHP-Ia who may be at risk for developing hypothyroidism. As the AHO phenotype generally is not clinically detected until mid-childhood, $G_s\alpha$ measurement may also be useful for screening young children in families affected with PHP-Ia in order to detect affected children before they become symptomatic.

The heterogeneous nature of $G_s\alpha$ mutations among pedigrees with PHP-Ia is not unexpected, as this pattern is common among genetic diseases that result from negative dominant mutations of the affected gene. The $G_s\alpha$ gene mutations that cause PHP-Ia may not be disseminated in the gene pool due to the severe endocrinopathies and mental impairment (11) that reduce the reproductive potential of affected patients. However, the variability of clinical symptoms and signs among PHP-Ia subjects detected during biochemical screening of PHP-Ia families (12) suggests that unknown environmental or genetic factors modulate the severity of clinical symptoms in patients with $G_s\alpha$ deficiency.

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