Parental origin of Gsα gene mutations in Albright’s hereditary osteodystrophy

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Abstract
Heterozygous mutations of the Gsα gene leading to reduced Gsα activity have been identified in patients with Albright’s hereditary osteodystrophy (AHO). However, AHO may be associated with hormone resistance (pseudohyoparathyroidism type Ia, PHP1a) or a normal response (pseudohyoparathyroidism, PPHP). As both disorders may occur within the same family, the relationship between Gsα genotype and phenotype remains unresolved. The AHO phenotype may be dependent upon the sex of the parent transmitting the Gsα mutation, perhaps through a gene imprinting mechanism. We have used an intragenic Gsα FokI polymorphism to determine the parental origin of Gsα gene mutations in sporadic and familial AHO. We now show that a de novo G→A substitution at the exon 5 donor splice junction in a child with PPHP was paternally derived. Furthermore, in a female with PPHP, the Gsα abnormality was shown to be of paternal origin, while subsequent maternal processing and transmission resulted in PHP1a in two offspring. As transmission of PPHP has rarely been reported, determining parental origin of the disease allele in sporadic cases may provide insight into the mechanism of hormone resistance in AHO.

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Albright’s hereditary osteodystrophy (AHO) is an autosomal dominantly inherited syndrome of short stature, obesity, round face, brachymetaphalangism, subcutaneous ossification, and variable mental retardation. Many subjects with AHO have associated pseudohyoparathyroidism type I (PHP1), failing to increase urinary cAMP and phosphate excretion after exogenous PTH.1 Resistance to other adenylyl cyclase stimulating hormones including TSH, LH, and FSH is frequently also observed.2 The remaining subjects, who respond normally to exogenous PTH, have pseudohypoparathyroidism (PPHP).4

Upon extracellular binding of many hormones to their specific receptors, an intracellular heterotrimeric G protein is activated. G proteins comprising α, β, and γ subunits can either stimulate (Gs) or inhibit (Gi) the formation of cAMP through adenylyl cyclase.5 The majority of patients with PHP1a have a 50% reduction in Gsα levels in membranes from various tissues including erythrocytes and renal cortex and these patients are classified as PHP1a. Interestingly, levels of Gsα activity are similarly reduced in patients with PPHP.6

The human Gsα gene has been mapped to chromosome 20q13,7 is 20 kb in length, and has 13 exons.8 Heterozygous Gsα gene mutations, either single base pair substitutions or small deletions of up to 43 bp,9,10 have been identified in patients with AHO. Within five separate kindreds, related persons with PHP1a and PPHP have been observed to cosegregate with the same Gsα mutation.11,12 Hence, molecular heterogeneity of Gsα mutations is not sufficient to explain the phenotypic difference in hormone responsiveness seen in PHP1a and PPHP.

A recent review of published familial cases of AHO14 suggests that parental origin of the mutated allele may influence phenotype since 66/66 offspring with PHP1 resulted from maternal transmission and 6/6 cases with PPHP resulted from paternal transmission. Such effects may be the result of genomic imprinting. Extensive studies in the mouse have led to identification of chromosome regions involved in imprinting.15 The mouse homologue of Gsα (GNAS) has been localised to distal chromosome 2 (2E1-2H3),16 a region known to show imprinting effects. In a further series of four families, we have noted phenotype concordance in all nine sibs, as predicted by an imprinting hypothesis (L Wilson, unpublished observations). A single instance of paternal transmission to an offspring with PHP1a has recently been described,17 but the Gsα mutation in this family has not been characterised.

In view of the paucity of reported male transmissions of AHO, we sought to test these observations in two persons with sporadic PPHP using a coding sequence polymorphism in order to determine the parental origin of the Gsα disease alleles.

Subjects and methods
PATIENTS
AHO 6 (III-1) (fig 1), is a 14 year old girl with short stocky build (height < 3rd centile, weight 50th centile), a round face, subcutaneous calcification, marked brachymetaphalangism, developmental delay, and grand mal fits. Her only medication is Carbamazepine. She was born after an uneventful pregnancy at 37 weeks’ gestation by emergency caesarian section for fetal distress and footling breech presentation and weighed 2100g. The neonatal course was complicated by bilateral pneumothoraces. At 2 weeks she was noted to have
abnormal renal function (urea 6-7 mmol/l, NR 2-8-6; creatinine 122 μmol/l, NR 42–74; glomerular filtration rate 16 ml/min/1.73 m²). Renal imaging by ultrasound showed diethylene triamine penta-acetic acid (DTPA) scan, intravenous urogram, and cystogram was normal. At 14 years of age, all serum calcium levels have been normal but her renal function remains impaired complicating assessment of her endocrine status (urea 7-7 mmol/l (NR 2-8-6), creatinine 121 μmol/l (NR<102), Ca 2-35 mmol/l (NR 2-22–2-58), P1 0-01 mmol/l (NR 0-9–1-38), Mg 0-52 mmol/l (NR 0-73–0-99), basal intact PTH 53 ng/l (NR 5-45)). However, thyroid function is normal (T4 78 nmol/l (NR 65–165), TSH 2-9 mU/l (NR 0-5–5)) and intravenous PTH infusion testing at the age of 4 years produced a normal urinary cAMP and phosphaturic response (table). Her four younger siblings are clinically normal. Erythrocyte G6P bioactivity was reduced to 76% of the normal control compared with 126% and 127% respectively in her clinically normal father and mother. These findings are compatible with a diagnosis of PPHP.

AHO 2 (II-2) (fig 2) is a 22 year old female with short stature (<3rd centile), marked shortening of the fourth and fifth metacarpals and distal first phalanges, but no ectopic calcification. She is normocalcaemic (Ca 2-45 mmol/l (NR 2-22–2-58), P1 0-08 mmol/l (0-9–1-38), basal intact PTH 10 ng/l (NR 5-45)) with normal urinary cAMP and phosphaturic responses to PTH (table). Currently she is treated with Carbimazole for hyperthyroidism (free T3 16-5 pmol/l (NR<8-6), positive antithyroid microsomal antibody titre 1:100 000). Her erythrocyte G6P activity was...

**Figure 1.** The upper photograph shows the DGGE analysis of exon 5, identifying the FokI genotype and the sequence variation in subject II-1 from AHO 6. The lower photograph shows the FokI digest on PCR products of the same persons on a 2% agarose gel. The genotypes of the subjects are shown between the photographs. The sequences of the oligonucleotide primers used for amplification of exon 5 are: 5’GGGCCGAGGCTCCCCGCCCCGGGGGCGG- CGCCGAGGGCGCCCCGAGGCAGCTCTTTCTCAC- GACCCG 3’ (forward primer) and 5’CTATATGGAAC- CTGCTCTCGAG 3’ (reverse primer). Comparison of the two figures showed that homoduplex band 2 arises from the FokI (−) allele and homoduplex band 1 from the FokI (+) allele. The DGGE analysis also showed that the variant pattern in II-1 is present on the paternally derived allele.

**Results of PTH infusion tests**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Basal urinary cAMP/Cr</th>
<th>Peak urinary cAMP/Cr</th>
<th>Basal urinary Pi/Cr</th>
<th>Peak urinary Pi/Cr</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHO 6, II-1</td>
<td>0.4</td>
<td>0.90</td>
<td>1.82</td>
<td></td>
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<tr>
<td>AHO 2, II-2</td>
<td>0.3</td>
<td>0.65</td>
<td>2.9</td>
<td></td>
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<tr>
<td>AHO 2, III-1</td>
<td>0.67</td>
<td>1.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PTH infusion testing was performed using a bolus of synthetic PTH-(1-34) (Rorer pharmaceuticals) at 3U/kg in 10ml of diluent (Rorer pharmaceuticals). Basal levels are the mean urinary cAMP (μmol/l) to urinary creatinine (mmol/l) ratios and urinary phosphate (mmol/l) to creatinine ratios for two 30 minute urine samples taken in the hour before administration of PTH. Following PTH infusion urine samples were collected at 30 minute intervals for three hours and the maximum cAMP and phosphate to creatinine ratios achieved are recorded. Normal responses are a greater than six fold rise in cAMP/Cr and three fold rise in Pi/Cr.

**Figure 2.** Results of DGGE analysis of exon 5 in family AHO 2. Bands 1 and 2 represent the homoduplexes of the (+) and (−) alleles respectively and bands 3 and 4 the heteroduplexes from the (+) and (−) alleles. Filled symbol denotes the PPHP phenotype and the shaded symbol denotes the PPHP phenotype.
Reduced to 54%. These data support a diagnosis of PHP.

Her mother, I-2, is clinically normal with Gsa activity of 110%. Her dead father was of normal stature with no known endocrine disorder.

The proband’s daughter, III-2, was induced at term. The neonatal period was complicated by the development of necrotising enterocolitis on the second day. Bradycardia was noted soon after birth and proved secondary to hypothyroidism (T4 47 mmol/l; NR 90–195), TSH>30 mIU/l (NR 0.5–6) Thyroid microsomal antibodies were weakly positive. Transient hypocalcaemia (Ca 1.83 mmol/l (NR 2.2–2.6)) was documented. Now aged 4 years she has a round face, obesity, developmental delay, and brachymetaphalangism but no ectopic calcification. She has subsequently remained normocalcaemic but had a markedly blunted urinary cAMP and phosphate response to intravenous PTH (table). Erythrocyte Gsa levels are reduced to 60% of a normal control. These clinical and metabolic features are compatible with a diagnosis of PHP type I. The proband’s son, III-1, also presented with hypothyroidism in the neonatal period. At 1 year he has a round face, stocky build, and developmental delay. He has remained normocalcaemic and has not been assessed by PTH infusion testing. His Gsa levels are reduced to 64% of a control.

Measurement of Gsa Bioactivity
Two-five ml samples of venous blood in citrate anticoagulant were frozen on dry ice and stored at −70°C. Measurement of erythrocyte Gsa bioactivity was by ccc-reconstitution assay as described by Bourne et al and membranes were kindly provided by Dr C Van Dop. Purification of [32P]cAMP was as described by Salomon et al using Dowex Alumina chromatography and [3H]cAMP to monitor recovery. Sample eluates were counted in 8 ml of scintillation fluid (Universol ES) in a dual channel scintillation counter. Values are the mean of duplicates corrected for cAMP recovery and expressed as a percentage of the [32P]cAMP production in a concurrent sample from an unrelated healthy control.

PCR Amplification of Genomic DNA
Genomic DNA isolated from peripheral leucocytes was used for amplification in a 50 µl reaction mixture containing deoxynucleotides (200 µmol/l each), forward and reverse oligonucleotide primer (1 µmol/l each), 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mmol/l MgCl2, and 1 U Taq DNA polymerase (Applied Biotechnologies). Amplification consisted of denaturation at 93°C followed by 35 cycles of 93°C for one minute and 60°C for 30 seconds and a final primer extension of two minutes at 70°C. The PCR products were analysed on 2% agarose gels, stained with ethidium bromide, and visualised by UV light. Sequences for the oligonucleotide primers were obtained from L S Weinstein and are mentioned in the legend to fig 1.

DGGE Analysis of PCR Amplified DNA Fragments
PCR amplified DNA fragments were analysed by DGGE as described previously. The samples were subjected to electrophoresis for 5-5 hours at 150 V and 60°C in an 8% polyacrylamide gel containing a gradient linearly increasing from 40 to 70% (100% denaturant is 7 mol/l urea and 40% (v/v) formamide). After electrophoresis, the DNA fragments were visualised by ethidium bromide staining.

FokI Coding Sequence Polymorphism Analysis
A FokI polymorphism present within exon 5 was confirmed as follows: a 10 µl aliquot of exon 5 amplified product was digested with 0.8 U FokI (New England Biolabs) in the recommended buffer for one hour at 37°C and analysed on 2% agarose gels.

DNA Sequencing
PCR amplified DNA fragments were sequenced using a PCR based assay. Gel purified fragments were dissolved in 10 µl H2O. Asymmetrical PCR was performed using 1 µl of the purified fragment, 75 µmol/l deoxynucleotides, and 1 µmol/l forward or reverse primer for 15

Figure 3  Sequence analysis of exon 5 and flanking regions in family AHO 6(A) in I-1, the unaffected father, (B) in II-1, and (C) of the paternally derived allele in II-1. This identified that a G→A substitution has arisen at position +1 of intron 5 on the paternally derived allele.
cycles as described, followed by another purification, after which the DNA was dissolved in 5μl H₂O. The generated template was subsequently used in cycle sequencing, using primers end labelled with [γ-32P]dATP (Amersham) and DNA Taq polymerase. The products were mixed with an equal volume of formamide loading dye (95% formamide and 20mmol/l EDTA), heat denatured, and run on an 8% denaturing polyacrylamide gel at 50W for two to four hours, followed by x-ray autoradiography.

Results
PARENTAL ORIGIN OF A G→A SUBSTITUTION IN AHO 6
Analysis of PCR amplified products encompassing exon 5 by DGGE detected sequence variation within this exon and genotypes were confirmed by FokI restriction analysis. Comparison of DGGE and restriction fragment analyses allowed assignment of homoduplex bands (fig 1). The father (I-1) and mother (I-2) of the proband (II-1) are homozygous negative and positive respectively for the diallelic FokI polymorphism. The proband was confirmed by FokI restriction fragment analysis to be heterozygous. However, on DGGE analysis she was found to have an altered banding pattern compared with all other heterozygotes indicating the presence of a DNA sequence alteration within this amplified fragment. Furthermore, the absence of a paternally derived homoduplex confirmed that the mutation had arisen on the paternal allele. Direct sequencing of amplified genomic DNA encompassing exon 5 from the patient II-1 showed a heterozygous G→A substitution (fig 3B) which was not present in either parent.

To confirm the parental origin of this Gsα mutation, the PCR amplified exon 5 fragment in II-1 was digested with FokI. The minus (−) allele was purified from an agarose gel and sequenced to show the presence of the mutation (fig 3C). The de novo origin of the mutation was confirmed through multilocus DNA fingerprinting for paternity (data not shown). The G→A substitution was not observed in 78 normal chromosomes.

PARENTAL ORIGIN OF THE MUTANT GSα IN AHO 2
PCR products encompassing exon 5 were subjected to DGGE. The analysis (fig 2) showed the family to be informative for the FokI polymorphism. Genotyping of affected subjects II-2, III-1, and III-2 showed cosegregation of the (−) allele with the reduced Gsα activity and the AHO phenotypes indicating that this allele carries the Gsα mutation. The grandmother I-2 is homozygous for the (+) allele hence the mutant allele is grandpaternally derived. Thus within one family a paternal Gsα mutation has resulted in the PPHP phenotype, while subsequent maternal transmission has resulted in offspring with PHPla.

Discussion
This is the first report showing the parental origin of a Gsα gene mutation in sporadic AHO. This is of particular interest in the light of the proposal that genomic imprinting may explain the alternative phenotypes, PHPla or PPHP that result from identical Gsα mutations. Only six cases of paternal transmission have previously been reported. This may be because of reduced male fertility or alternatively ascertainment bias should these offspring have normal endocrine function. Under these circumstances, identifying the parental origin of Gsα mutations in sporadic PPHP cases is a valuable approach to testing this apparent parental effect on phenotype.

The proband in family AHO 6 has a classical AHO phenotype. The diagnosis of PPHP is based on her normal response to PTH infusion testing, normocalcaemia, normal thyroid function and reduced Gsα levels. While the presence of renal impairment in this subject would have caused difficulty in interpreting a blunted cAMP and phosphaturic response to exogenous PTH, it does not alter the significance of a normal response. We have identified a heterozygous G→A substitution within the consensus donor splice site at position +1 of intron 5 which is likely to result in loss of the donor splice site. In addition, we have shown that this mutation is paternally derived and has arisen de novo since paternity has been confirmed by DNA fingerprinting (data not shown). The FokI polymorphism results exclude the possibility of uniparental disomy.

In family AHO 2, subject II-2 has unequivocal PPHP based on her AHO phenotype, normal response to exogenous PTH, and reduction in erythrocyte Gsα levels. While the de novo nature of the AHO cannot be confirmed since her father is dead, an imprinting hypothesis predicts equivalent outcomes from a paternally derived de novo mutation and a paternal transmission. Hyperthyroidism is not a typical feature of AHO and strongly positive antithyroid microsomal antibody titres in II-2 supports a coincidental autoimmune aetiology. Interestingly both her offspring have been described before in PHPla. The diagnosis has been confirmed in III-2 by the blunted response to exogenous PTH and the reduced erythrocyte Gsα levels. Our data show the “switch” in phenotype is dependent on the sex of the transmitting parent across two generations.

There is accumulating evidence that genomic imprinting plays a role in human disease. Genomic imprinting of the Gsα gene alone would not explain the clinical observations in AHO as equivalent reductions in Gsα bioactivity are found in PHPla and PPHP in all tissues tested to date. However, imprinting at a tissue or even cellular specific level could account for these findings. A simple model would require that cells such as renal tubular cells express only the maternal Gsα allele while other cells such as erythrocytes express both alleles. A mutation in the maternal allele would result in renal resistance to PTH. Elsewhere a mutation in either parental allele would result in a 50% reduction in measured Gsα activity, giving rise to the AHO phenotype.
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Gsα mutation would not be expressed in the implanted cells resulting in no observed PTH resistance and hence PPHP. Of note, the finding of Downs et al.9 that Gsα levels in the total renal cortical membranes of a patient with PHP1 were 30 to 40% of controls is compatible with a cellular specific imprinting model. Tissue specific imprinting has been reported in the mouse IGF2 gene where only the paternal allele is expressed in all embryonic tissues except the choroid and leptomeninges where both parental alleles are expressed.31

PTH responsive cells suitable for direct testing of a cell specific imprinting hypothesis are not readily accessible and no animal model of AHO has been identified. At present, evidence for imprinting is derived solely from clinical observation of parental effects on phenotype. We have used a molecular genetic strategy to identify two additional instances of paternally derived Gsα abnormalities causing PPHP and shown in one of these that subsequent maternal transmission has resulted in PPHPa. Clarification of the nature of any parental effect on phenotype will be of particular significance for accurate genetic counselling in AHO as well as understanding the pathological mechanisms responsible for development of the disorder.

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