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DIFFICULT CASE

A Novel, Complex Heterozygous Mutation Within Gsα Gene in Patient with McCune-Albright Syndrome

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McCune-Albright syndrome (MAS) is caused by embryonic somatic mutations leading to the substitution of His or Cys for Arg at amino acid 201 of the α-subunit of the signal transduction protein Gs (Gsα). The mutations have been found in many affected tissues of patients with MAS. Recently, a new missense mutation was detected in a patient with MAS, leading to the substitution of glycine for arginine at amino acid 201 of the Gsα gene, whereas no mutations have been reported at other sites in this gene. In the present study, we identified the activating mutations in the gene encoding Gsα protein in the osseous lesions of fibrous dysplasia and peripheral blood leukocyte in a 17-yr-old male patient with MAS. In addition, a heterozygous mutation encoding substitution of Arg201 of Gsα with His was found. Interestingly, we also found the other two types of mutations within the Gsα gene in the patient's affected osseous tissue. One is a combination mutation in the same allele at codons 209 and 210 of the Gsα gene, and the other the missense mutation at codon 235.

Key Words: McCune Albright syndrome; Gsα gene; gene mutation.

Introduction

First reported by McCune *(1)* in 1935 and shortly thereafter by Albright et al. *(2)*, McCune-Albright syndrome (MAS) is an uncommon clinical disorder characterized by the tetrad of polyostotic fibrous dysplasia, cutaneous hyperpigmentation, and some hyperfunctional endocrinopathies, such as sexual precocity, GH excess, hyperthyroidism, and hypercortisolism *(3–5)*.

The pathogenesis of this peculiar syndrome affecting multiple systems has remained unknown for many years. By examining a 4-yr-old female patient and reviewing the photographs published in the literature, Happle found that the cutaneous pigmentation displayed a typical segmental distribution frequently following the lines of Blaschko, reflecting an underlying mosaicism. He proposed that MAS resulted from a postzygotic somatic cell mutation *(6,7)*.

Recent evidence has shown that the clinical manifestations of MAS are caused by embryonic somatic mutations leading to the substitution of His or Cys for Arg at amino acid 201 of the α-subunit of the signal transduction protein Gs (Gsα) *(8,9)*. The mutations in the gene coding for the Gsα protein have been found in many affected tissues of patients with MAS, including the skin, bone, pituitary, thyroid, adrenal gland, testis, ovary, and other nonendocrine tissues *(8–14)*. The mutations inhibit the guanosine-5'-triphosphatase (GTPase) activity of Gsα, and adenylate cyclase is constitutively activated. The clinical characteristics are postulated to be caused by autonomous signaling in tissues, such as those of endocrine organs, which express a Gsα-linked signaling mechanism. Identification of Gsα mutations may help to define a more complete clinical spectrum of MAS.

In the present study, we identified the activating mutations in the gene encoding Gsα gene in the osseous lesions of fibrous dysplasia and peripheral blood leukocyte in a 17-yr-old male patient with MAS, and we found an unusual complex of heterozygous mutations in the Gsα gene in this patient.

Results

Case Report

The patient, a 17-yr-old Chinese male high school student, had been suffering from recurrent pathologic fractures following minor traumas. At the age of 12, he sought medical attention for chronic nasosinusitis and a diagnosis of polyostotic fibrous dysplasia was made by radiograph. Pubertal development was normal. His parents are nonconsanguineous and of average height, and there is no family history of similar bone diseases or any endocrinopathy. Before age 14 he was of average height for his age group. During the past 3 yr, his height has increased at a rate of 10 cm/yr, and he has become much taller than his peers. On

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physical examination, the patient's height was 182 cm and weight was 70 kg. Multiple, large café-au-lait lesions with irregular borders were present over the skin and mucosa of the labium maxillae and labium inferius oris (Fig. 1A–B). His face was slightly asymmetric as a result of prominent right ossa malare.

Radiologic examination showed multiple bone lesions characteristic of polyostotic fibrous dysplasia in the humera, femurs, ilia, ribs, and skull (Fig. 1C). ^{99}TC-MDP scanning of the skeleton revealed high radioactive uptake at multiple areas of bone lesions (Fig. 1E). Computed tomography (CT) and magnetic resonance imaging (MRI) of the brain demonstrated bony frontotemporal overgrowth affecting ethmoidal and sphenoidal bones. The pituitary was normal (Fig. 1D). Visual fields appeared normal. Histologic examination of bone showed fibrous dysplasia (Fig. 2).

Serum and urinary concentrations of phosphate and calcium were normal. Serum alkaline phosphatase was elevated to 1942 IU/L (reference value: 42–121 IU/L), and urinary pyridol was elevated to 19.9 nmol/mmol of urinary creatinine. Plasma concentrations of levorotatory thyroxine (T_4), triiodothyronine (T_3), free T_4, free T_3, thyroid-stimulating hormone (TSH), cortisol, adrenocorticotropic hormone (ACTH), testosterone, E_2, follicle-stimulating hormone, luteinizing hormone, and leptin were normal. Serum levels of growth hormone (GH) (15.3 and 14.3 ng/mL; normal range: 0–10 ng/mL), parathyroid hormone (PTH) (75.3 and 238 pg/mL; normal range: 13–53 pg/mL), and prolactin (PRL) (19 ng/mL; normal range: 2.9–17.1 ng/mL) were slightly elevated.

Polymerase Chain Reaction Amplification and DNA Sequencing

Amplification of genomic DNA with primers produced a 375-bp fragment that included exons 8 and 9 of the Gsα gene. The bands were purified using the Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturer's recommendation and subcloned to PGEM-T easy vector (Promega, Madison, WI). Ten and four clones were selected from osseous tissue and leukocytes, respectively, and sequenced by BigDye Terminator Cycle Sequencing Ready Reaction Kits (Perkin-Elmer) on an ABI 377 DNA sequencer.

The results revealed that three clones from the patient's affected bone and one clone from leukocytes presented a single C-to-T transition within the codon for Arg201. This mutation changes the normal CGT to TGT and results in the substitution of cysteine for arginine (Arg201 → Cys201). The DNA sequence analysis of the other clones revealed the wild-type sequence at this position (Fig. 3A). Interestingly, two types of mutations were newly found within the Gsα gene amplified from the patient's affected osseous tissue. One type consisted of the combination mutation in the same allele at positions 209 and 210 of the Gsα gene. Sequencing showed the presence of a single A-to-G and C-to-T transversion in the same clone at positions 209 and 210, respectively (Fig. 3B), leading to Glu209Gly and Thr210Ile substitution. The other was a mutation at position 235 with a single A-to-G transition (Fig. 3C), leading to Ile235Val substitution. These two type of mutations were not found in exons 8 and 9 of DNA amplified from the patient's leukocytes. The presence of both normal and mutant sequences of the Gsα gene indicates the occurrence of a somatic mutation and thus a mosaicism of normal and abnormal cells as observed in the case of MAS.

Confirmation of Two Types of Novel Mutations

To exclude the possibility of polymerase chain reaction (PCR) amplification errors or polymorphism for the newly found mutations at these positions, the fragments containing codons 209 and 210 and those including codon 235 were amplified by pair primers 1 and 2, respectively, as described in the Materials and Methods, from the genomic DNA extracted from the leukocytes of 10 normal subjects and the patient's leukocytes and osseous tissue and were digested by *Alw*I. As shown in Fig. 4, the fragments containing codons 209 and 210 amplified from peripheral leukocytes of control subjects and the patient produced one fragment of 201 bp, but the combination mutation made the fragments amplified from the genomic DNA extracted from the patient's affected bone with one *Alw*I site and thus generated two fragments of 142 and 59 bp. The fragments containing codon 235 from the genomic DNA extracted from peripheral leukocytes of control subjects and the patient were cut at two *Alw*I sites, producing three fragments of 159, 73, and 9 bp, whereas the missense mutation at position 235 made the fragments amplified from genomic DNA extracted from the patient's affected bone with one *Alw*I site, thus generating only two fragments of 232 and 9 bp. The fact that these mutations were found in the patient's affected bone but not in leukocytes of the patient and normal subject indicates that these mutations are not polymorphic.

The 36 clones selected from the patient's affected osseous tissue were amplified by the two pair primers as already mentioned, then digested by *Alw*I. As a result, a combination mutation in the same allele at positions 209 and 210 of the Gsα gene was found in one clone, and a missense mutation at position 235 was detected in two clones (data not shown).

Discussion

MAS is a sporadic disease characterized by polyostotic fibrous dysplasia, café-au-lait spots, and various endocrine disorders, including precocious puberty, hyperthyroidism, hypercortisolism, GH excess, and hyperprolactinemia. The diverse metabolic abnormalities seen in MAS are related to the involvement of cells that respond to extracellular signals through activation of the hormone-sensitive adenylate cyclase system *(9)*. In 1991, Weinstein et al. *(8)* first reported a missense mutation at codon 201 of the gene encoding the

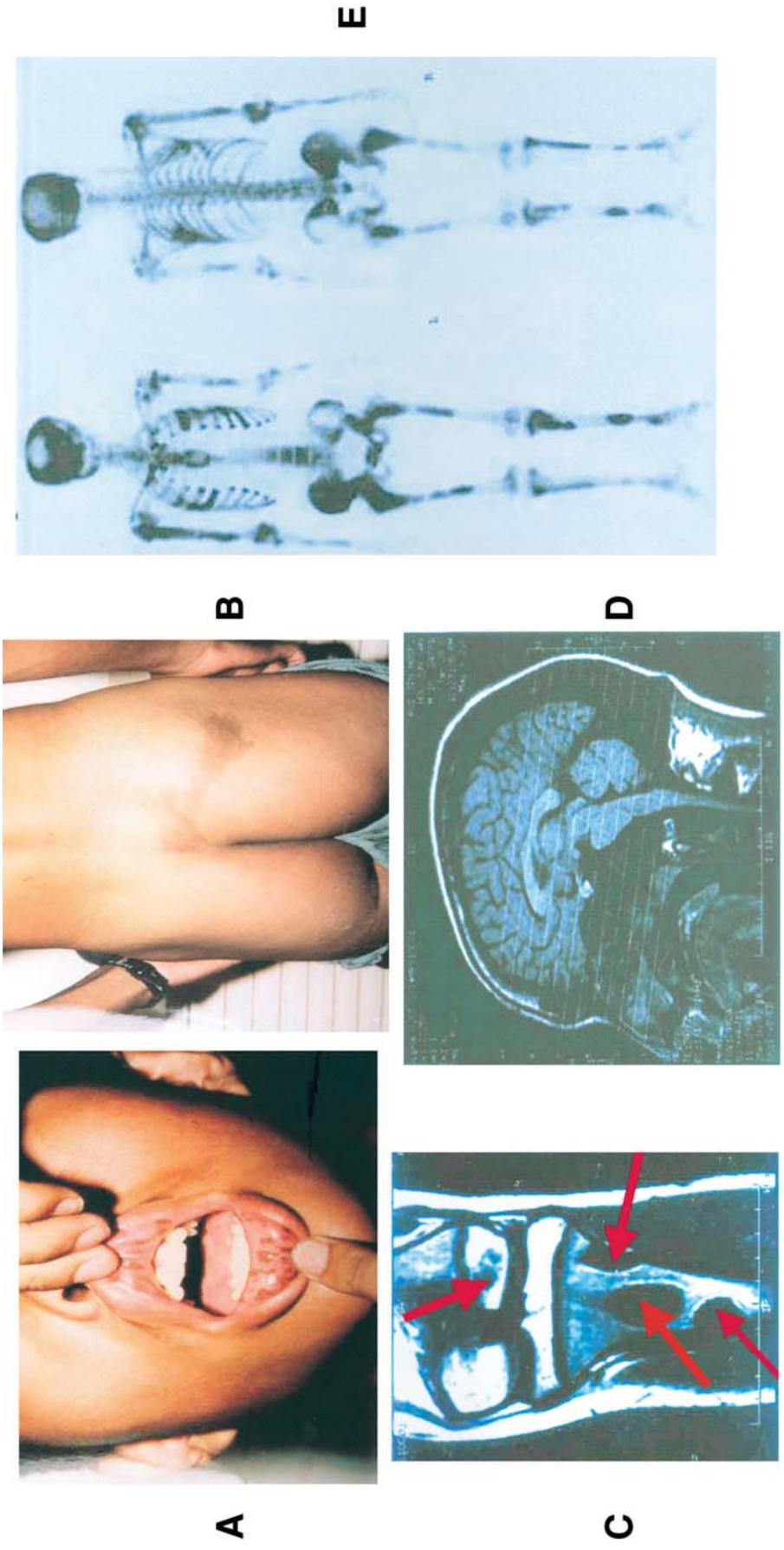

Fig. 1. Skin and bone lesions in patient with MAS. (**A**,**B**) Multiple café-au-lait lesions with irregular borders over mucosa of labium maxillae and labium inferius oris, and over skin of right buttock; (**C**) polyostotic fibrous dysplasia involving left tibia; (**D**) MRI of sella showing normal size of pituitary, and bony frontotemporal overgrowth involving ethmoidal and sphenoidal bones; (**E**) 99TC-MDP scanning of bone revealing high radioactive uptake on multiple areas of skeleton.

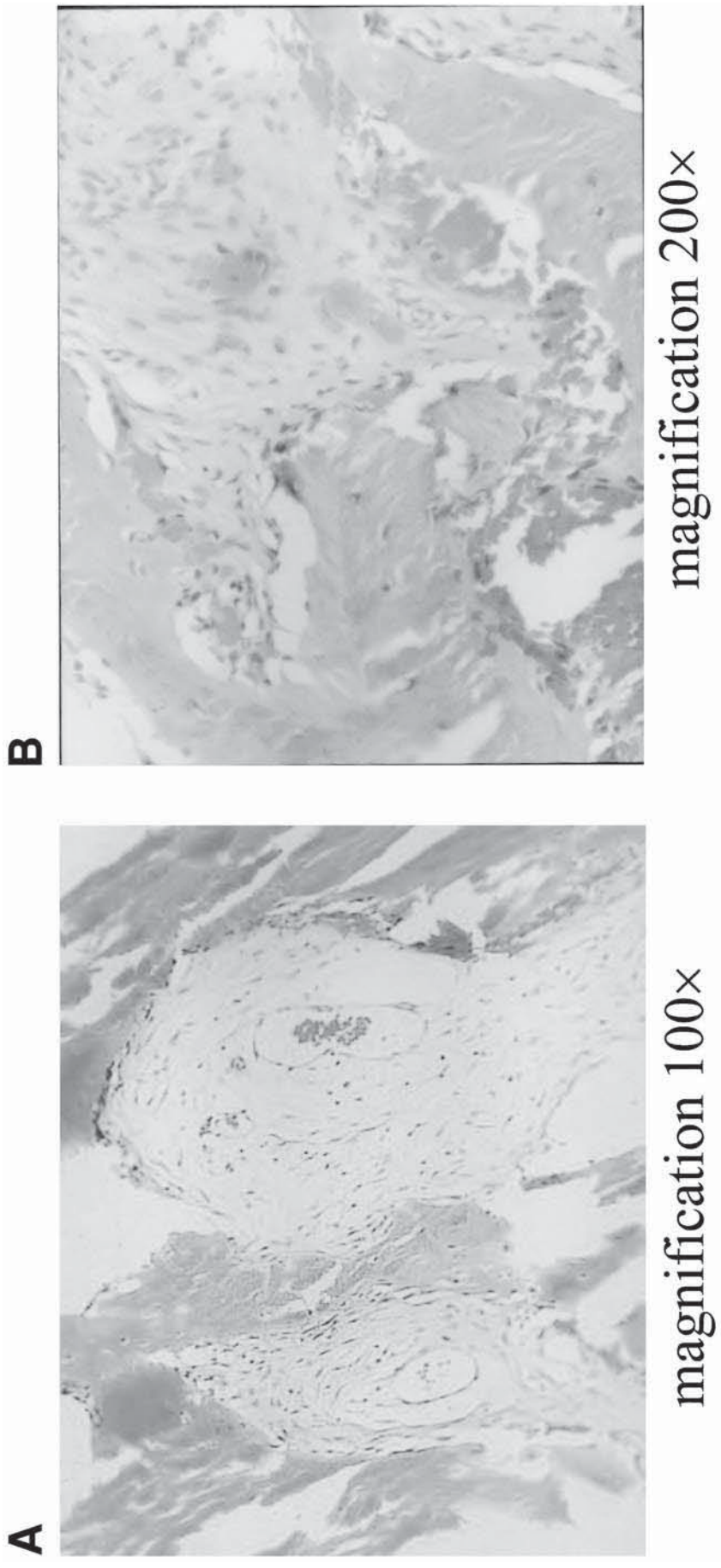

Fig. 2. (**A**,**B**) Photomicrographs of hematoxylin and eosin–stained bone sections from patient with MAS showing irregularly woven bone trabeculae being formed with a cellular fibroproliferative background. The newly formed bony spicules follow the swirled pattern of the fibrous tissue. Proliferative fibroblasts appear spindle shaped. (**A**): magnification = ×100; (**B**): magnification = ×200.

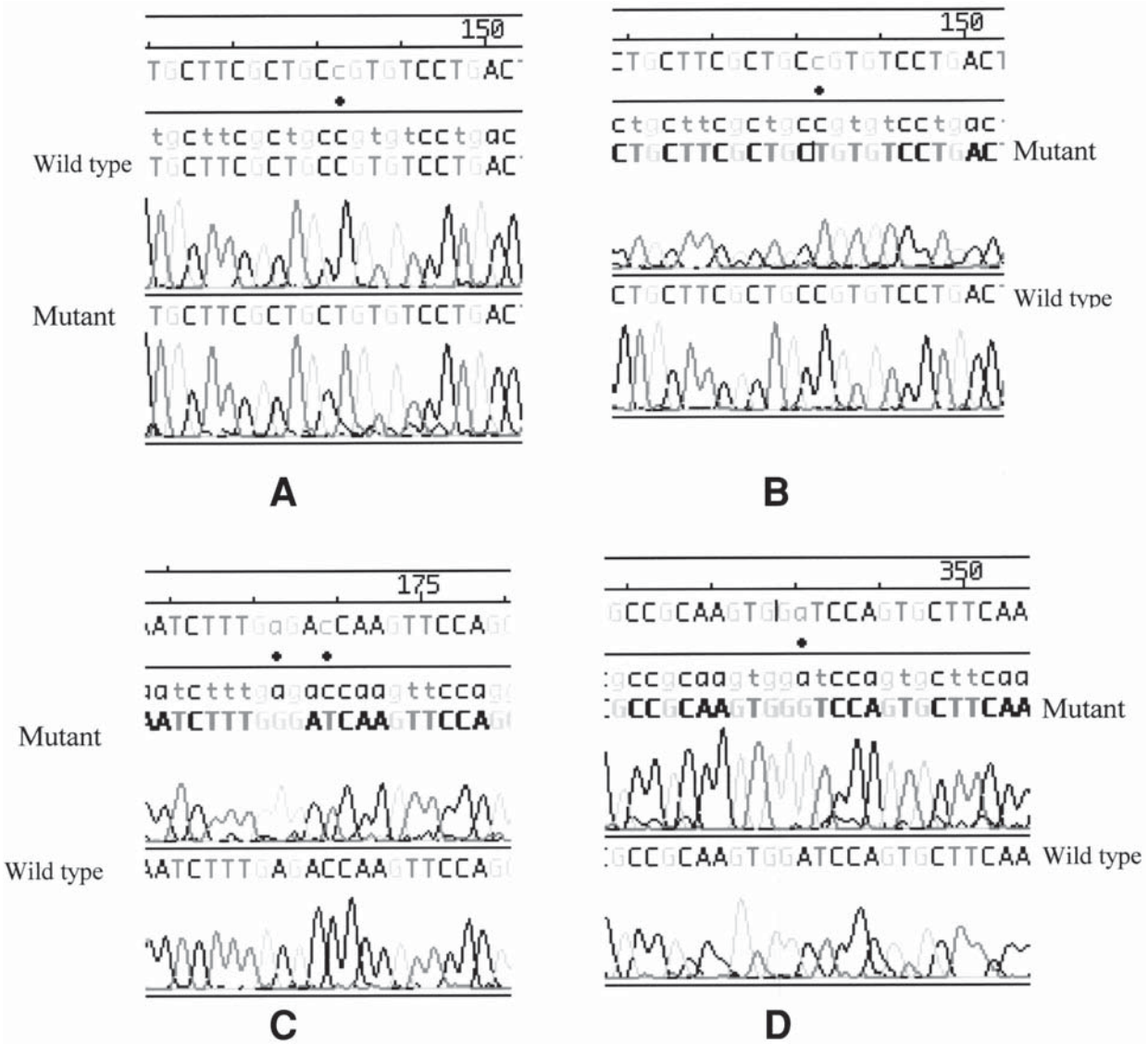

Fig. 3. DNA sequence analysis. (**A,B)** Fragments amplified from patient's affected bone and leukocytes, respectively. Note the C-to-T transition in the codon for Arg^{201}. (**C,D**) The regions of interest of Gsα were amplified DNA extracted from the patient's affected bone. (**C**) Combination mutation in the same allele at the codons 209 and 210 of the Gsα gene. Note a single A-to-G and C-to-T transversion at codons 209 and 210, respectively, leading to Glu209Gly and Thr210Ile substitution at codons 209 and 210. (**D**) Missense mutation at codon 235 on the Gsα gene. Note the single A-to-G transition in the codon for Ile^{235}.

α-subunit of the G protein in patients with MAS. Up to now, fewer than 200 patients with MAS have been reported. Among them, fewer than 40 patients were investigated for the mutation within the gene encoding the α-subunit of the G protein. Two gain-of-function somatic missense mutations have been found in all tissues affected in patients with MAS, arginine 201 with either a cysteine or histidine substitution *(8–14)*. Recently, a new missense mutation was detected in a patient with MAS, leading to the substitution of glycine for arginine at amino acid 201 of the Gsα gene *(15)*, whereas no mutations have been reported at other sites in the gene encoding the α-subunit of the G protein. The arginine 201 residue is critical for α-subunit GTPase activity, and each of the described mutations decreases GTPase activity of the Gsα subunit and leads to constitutive activation.

In the present study, a G-to-T transition was found within exon 8 of the Gsα gene amplified from the patient's affected osseous tissue and leukocytes. This single-base substitution results in the replacement of arginine with cysteine at position 201 of the mature Gsα protein. Interestingly, we also newly found two other types of mutations in the affected bone of the patient. One is a combination mutation in same allele at positions 209 and 210 of the Gsα protein, and the other the missense mutation at the position 235. However, these two novel mutations were not found in exons 8 and 9 of DNA from the patient's leukocytes. The results suggest

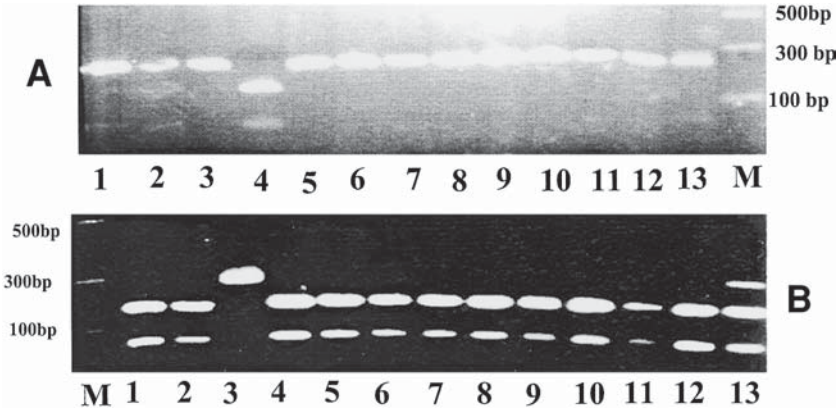

Fig. 4. Restriction enzyme analysis of fragments amplified from exon 8 or 9 of Gsα. M: DNA marker. (**A**) A 201-bp fragment amplified from exon 8 of Gsα with pair primers 1. The combination mutation in the same allele at positions 209 and 210 introduced a restriction site for *Alw*I, generated two fragments of 142 and 59 bp. Lane 1, amplified DNA from patient's blood; lane 2, DNA obtained from affected bone; lane 3, DNA obtained from wild-type clone; lane 4, DNA obtained from mutant clone; lanes 5–13, DNA obtained from normal subject's blood. (**B**) A 242-bp fragment amplified from exon 9 or 8 of Gsα with pair primers 2. The missense mutation at position 235 of the Gsα abolished a restriction site for *Alw*I, thus generating two fragments of 232 and 9 bp. When no mutation was present, three fragments of 159, 73, and 9 bp were yielded. Lane 1, DNA obtained from patient's blood; lane 2, DNA obtained from wild-type clone; lane 3, DNA obtained from mutational clone; lanes 4–12, DNA obtained from normal subject's blood; lane 13, DNA obtained from affected bone of patient with MAS.

that MAS in this patient is caused by a novel, complex heterozygous mutation at the gene encoding the α-subunit of G protein.

The highly conserved regions of three switches within the Gsα protein are required for the activation of adenylate cyclase by Gsα *(16)*. The nucleotide is bound within the narrow cleft formed by switch I (codons 199–207) and switch II (codons 244–240) *(17)*. These suggested mutations near switch I (codons 209 and 210) and within switch II (codon 235) probably have an effect on the interaction between Gsα and adenylate cyclase.

In our case patient, serum levels of GH and PRL were elevated, but a pituitary tumor could not be detected by CT and MRI scans. This is not surprising because evidence of a pituitary adenoma was found in only 64% of MAS patients with GH excess by Chanson et al. *(18)*. There may be two reasons for the result. One possibility is that skull thickening compromises the interpretation of CT scans, but MRI is sufficient to distinguish clearly adenomatous pituitary and fibrous bone tissue *(18)*. Another possibility is that because the pituitary adenoma is at its early stage, it is so small that it cannot be detected by CT or MRI scan. According to the literature, in almost all MAS patients of tall stature, GH excess was associated with hyperprolactinemia *(19)*, as in our patient.

In contrast to sporadic endocrine tumors, the Arg 201 mutation on the Gsα gene in MAS patients is present in multiple tissues and thus leads to various manifestations of the syndrome. It has been postulated that the MAS may result from a single early postzygotic somatic mutation, leading to a mosaic distribution of the mutation *(6–8)*, whereas in the case of solitary adenoma the somatic mutation occurs probably at a late stage and affects only one cell type. The ultimate phenotype in a patient with MAS bearing a Gsα mutation depends on the timing, location, and destination of the original cell containing the mutation. Whether a Gsα mutation could affect the differentiated phenotype of a cell also depends on the functional significance of Gsα in these cells. Very early somatic mutations would involve many tissue types and have a widespread distribution, whereas later mutations would have a more limited distribution and result in less variation from the normal phenotype *(8,9)*. The fact that the Arg201Cys mutation within Gsα gene occurs in both leukocytes and osseous lesions, whereas the two types of mutations newly found in the Gsα gene were detected only in osseous lesions, suggests that two independent events occurred in the process of the patient's embryonic development: an early postzygotic somatic mutation (the Arg201Cys mutation on the Gsα gene), and a later somatic mutation affecting only the bone (two types of novel mutations on the Gsα gene).

Materials and Methods

Subject

The patient and his family gave informed consent for this study.

Laboratory Assays

Plasma gonadal steroids, gonadotropic hormones, T_4, T_3, free T_3, and free T_4 concentrations were measured by immunoenzymatic method (Serono). Radioimmunoassays were used to measure the serum concentrations of cortisol, PTH, GH, and PRL (Diasorin). ACTH and TSH were analyzed

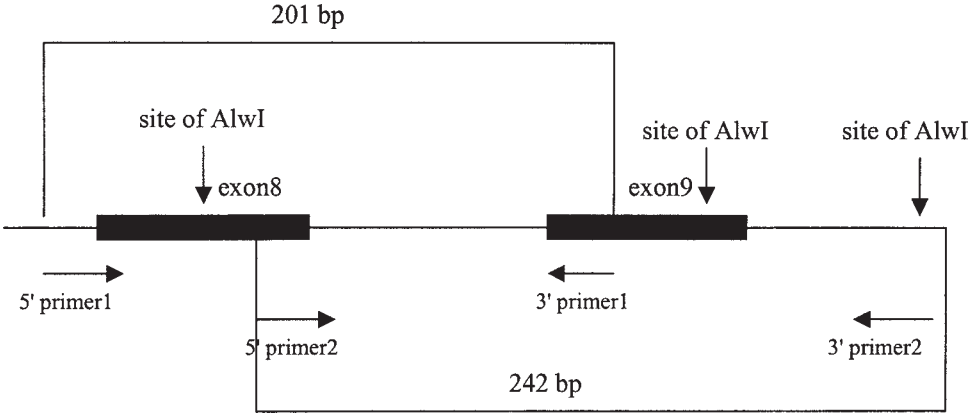

Fig. 5. Strategy for digesting regions of the Gsα gene with restriction enzyme *Alw*I. Horizontal arrows indicate the primers for PCR amplification. Vertical arrows indicate restriction enzyme sites of *Alw*I.

by immunoradiometric assay (Diagnostic System). Urinary deoxypyridinoline crosslinks were measured by competitive enzyme immunoassay (Metro Biosystems).

Amplification of DNA by PCR

Genomic DNA was isolated from leukocytes by protease-K digesting and phenol-chloroform extracting *(20)*. The osseous tissue in the lesion of the right ilium was obtained by harpoon biopsy under CT and immediately frozen in liquid nitrogen. The tissue was rapidly homogenized by milling in liquid nitrogen and then digested with 0.5 mg/mL of proteinase K (Sigma, Louis, MO), in the digestion buffer (50 mmol/L of Tris, pH 8.5; 1 mmol/L of EDTA; 0.5% Tween-20) for 16 h at 37ºC or 3 h at 50ºC. The sample was extracted three times with a 1:1 mixture of phenol and chloroform/isoamyl alcohol (24:1). The DNA was precipitated and washed by ethanol.

One pair of PCR primers was designed by Express Primer™1.0 software for amplifying exons 8 and 9 of the gene encoding the Gsα protein. The oligonucleotide primers with sequence identical to the introns flanking exons 8 and 9 of the Gsα gene were synthesized as follows: upstream, 5' CCAGACCTTTGCTTTAGATTGGC 3'; downstream, 5' CCCTGATCCCTAACAACACAGAAG 3'. PCR reaction mixtures (50 µL) contained 20 pmol of each primer, 500 ng of genomic DNA,200 µ*M* dNTPs, 1.5 m*M* $MgCl_2$, 2.0 U of AdvanTaq DNA polymerase (Clontech), 50 m*M* KCl, and 10 m*M* Tris-HCl at pH 8.3. A Perkin-Elmer thermal cycler 9600 was used to perform primary denaturation for 5 min at 95ºC, and then 30 cycles of denaturation for 45 s at 95ºC, annealing for 55 s at 57ºC, and extension for 1 min at 72ºC. The total final extension was 10 min at 72ºC.

DNA Sequencing Analysis

PCR fragments amplified from genomic DNA were analyzed by electrophoresis by 1.5% agarose gel and visualized with UV light after being stained with ethidium bromide. Optimal results of the PCR amplification were purified by means of Qiagen Quick column (Qiagen) and subcloned in PGEM-T easy vector (Promega). Plasmid DNA was prepared from 40 isolated clones (36 and 4 from osseous tissue and leukocytes, respectively) by alkaline lysis miniprep method. Ten and four double-strand plasmids DNA were selected from the patient's osseous lesion and leukocytes, respectively, and sequenced from two directions. The sequencing reactions were performed on a 9600 Thermal Reactor (Perkin-Elmer) using a BigDye Deoxy Terminator Cycle Sequencing Kit (Perkin-Elmer) and then analyzed using an ABI 377 DNA Sequencer (Perkin-Elmer) *(21)*.

Restriction Enzyme Analysis

In this work, two types of mutations were newly found within the Gsα gene amplified from the patient's affected osseous tissue. One is a combination mutation on the same allele at codons 209 and 210 of the Gsα gene, and the other the missense mutation at codon 235. The occurrence of a combination mutation in the same allele (Glu209Gly and Thr210Ile) introduces an *Alw*I restriction endonuclease site in exon 8 of the Gsα gene, whereas the Ile235Val missense mutation abolishes an *Alw*I restriction endonuclease site from exon 9; thus, the presence of the two types of novel mutations can be verified by restriction enzyme analysis.

Two pairs of PCR primers were designed by Express Primer™1.0 software for amplifying the combination mutation in the same allele at positions 209 and 210, and the missense mutation at position 235, respectively (Fig. 5). The oligonucleotide primers were synthesized as follows: Pair primers 1 was for amplifying the mutation at positions 209 and 210 of the Gsα gene: upstream, 5' GACCTCAATTTT GTTTCAGGACCTG 3'; downstream, 5' CGTCAAACAT GCTGGTGGG 3'; Pair primers 2 was for amplifying the mutation at position 235: upstream, 5' GTGGACAAAGTC AACTTCCAGTAAGC 3'; downstream, 5' CCCTGATCC CTAACAACACAGAAG 3'. To exclude the possibility of PCR amplification errors or the presence of polymorphism for the newly found mutations at these positions, the frag-

ments containing codons 209 and 210 and those including codon 235 were amplified by pair primers 1 and 2, respectively, from the genomic DNA extracted from leukocytes of 10 normal subjects and the patient's leukocytes and osseous tissue and were digested with 3.0 U of the restriction enzyme *Alw*I (BioLabs) at 37ºC for 16 h and electrophoresed through 3% agarose gel. The restriction fragments were visualized by UV light after being stained with ethidium bromide.

To detect the frequency of the two types of novel mutations in the patient's osseous tissues, the fragments containing codons 209 and 210 and those including codon 235 were amplified by pair primers 1 and 2 as described, respectively, from 36 isolated plasmids selected from the patient's osseous tissue and digested by *Alw*I. The restriction fragments were analyzed as described.

Acknowledgments

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