

The Use of Real Time PCR Genotyping to Detect Activating GNAS Mutations in McCune-Albright Syndrome

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Abstract

Introduction: The McCune-Albright syndrome (MAS) is a genetic disease clinically characterized by the triad: bone fibrous dysplasia café-au-lait skin spots and endocrine hyperfunction, such as precocious puberty. MAS is due to activating mutations of GNAS, the gene encoding Gs alpha and mutations analysis of this gene could increase the definitive diagnosis of MAS and atypical and partial.

Objectives: To identify the p.R201H and p. R201C GNAS activating mutations in multiple tissues derived from patients with MAS using real time PCR genotyping.

Material and methods: Genomic DNA was isolated from blood from 31 patients (28 females) with typical and atypical forms of MAS. Skin, adrenal gland or bone tissue samples were also available from six different patients. Genotyping based on PCR real time assay using TaqMan probes was performed for identification of p.R201H and p. R201C GNAS mutations. Cloning and sequencing were used as assenting techniques.

Results: Using real time PCR genotyping, no mutations in GNAS were identified in blood samples of MAS patients, only in bone sample of a patient with a previously identified p.R201H. Cloning and sequencing from blood of this same patient revealed that 5/150 clones harboring the p. R201H.

Conclusion: The real time PCR genotyping proved to be efficient for the molecular diagnosis of MAS in affected patient's tissues. Advantages of this technique are rapidity, accuracy, it is generally easy to perform and could be used routinely. Nevertheless, optimization of GNAS detection mutation is still necessary to considerer this technique to earlier diagnosis of non-classical forms of MAS using peripheral blood.

Keywords: McCune-Albright; gsp mutations; Real time PCR genotyping

Introduction

McCune-Albright syndrome (MAS) is a rare genetic disease, with incidence estimated between 1/100,000 and 1/1,000,000 cases/year. The classic syndrome is clinically characterized by a triad of physical signs: café-au-lait pigmented skin lesions, polyostotic fibrous dysplasia, and pseudo-precocious puberty. Some MAS patients present only two features of the classic triad or one of these features and another endocrine disorder, such as hyperthyroidism, gigantism/acromegaly, autonomous adrenal hyperplasia and Cushing's syndrome [1-4]. GNAS maps on chromosome 20q13 and encodes for the stimulatory G protein (Gsa) that is required for the activation of adenylyl cyclase (AC) and generation of cAMP in many cell types. All the endocrine glands that are hyperactive in MAS have an autonomous secretion and have in common a response to extracellular signals by the AC-cAMP pathway and constitutionally elevated AC activity. Consistent with these findings, activating mutations in GNAS protein have been identified in patients with MAS [5,6]. Usually, these

mutations are de novo post-zygotic and virtually all represent substitutions at residue Arg 201 (usually p.R201C, p.R201H, and occasionally p.R201S and p.R201G) [7]. It is generally accepted that the GNAS mutation in MAS must have occurred early in development producing a mosaic pattern distribution of mutant cells [6]. However only patient's affected organs carry a significant percentage of mutant cells and these tissues are not always available (4). A low percentage of mutated cells may be present in peripheral blood and could be used in the identification of GNAS-activating mutations leading to earlier diagnosis in patients with partial forms of MAS [8-10]. Nevertheless, this is only realistic if an accurate and sensitive assay for mosaic mutations detection is available. Here, we evaluated the use of TaqMan PCR genotyping system for the detection of GNAS mutations in multiple tissues from patients with MAS.

Patients and Methods

Experimental subjects

The study population consisted of 31 patients with MAS followed at Hospital das Clínicas, School of Medicine, University of São Paulo

center's Division of Endocrinology. The clinical patient's features are described in Table 1. Peripheral blood specimens were obtained from all patients. Other tissue samples were available from six different cases: a) skin - one patient with classical triad; b) bone - 1 patient with bone fibrodysplasia and Café-au-lait spots and 2 patients with bone fibrodysplasia and precocious puberty (the p.R201H mutation was previously identified in heterozygosis in one of these bone samples [11]; c - adrenal gland, 1 patient with precocious puberty and Café-au-lait spots and 1 patient with bone fibrodysplasia and Café-au-lait spots. Informed consent was obtained from all patients or their legal responsible in accordance with institutional Ethical Committee.

Characteristics	Male	Female
Number of patients	29	2
Median age, yrs range	2.5 (0.8-18)	35.28 (18-74.8)
Precocious puberty	18	0
Café-au-lait spots	20	2
Bone fibrodysplasia	21	2

Table 1: Clinical features of 31 patients with MAS

DNA extraction

DNA from peripheral blood leukocytes, bone and adrenal gland was extracted using standard procedures. DNA from skin was obtained after biopsy punch and cell culture [12] with Wizard genomic DNA Purification kit following manufacturer's instructions.

R201C and R201H GNAS real time PCR genotyping assay

The GNAS1 p.R201C and p.R201H were individually genotyped by allelic discrimination in a Real Time 7500 equipment (Applied Biosystems, Foster City, CA) using mutation specific TaqMan approach. Genomic DNA from blood and, in some cases, from skin, adrenal gland, bone were used in reactions containing 10 µL TaqMan universal PCR master mix, 1 µL Assays-by-Design probe and primer mix (Table 2, Applied Biosystems) in a total volume of 20 µL. DNA from the p.R201H mutant bone sample was used a positive control [11]. Amplification conditions were a single cycle of 95°C for 10 min followed by 40 cycles of 95 C for 15 sec and 60 C for 1 min.

	Primers
Forward	CTTTGGTGAGATCCATTGACCTCAA
Reverse	AATCTTTGAGACCAAGTCCAGGTG
Reporter dye	Probe designed for the detection of the p.R201H
FAN -mutated allele	TCGCTGCCATGTCC
VIC - wild type allele	TCGCTGCCGTGTCC

Table 2: Designed primers and probes for the detection of the gsp mutation

Amplification, cloning and sequencing of GNAS1 from peripheral-blood leukocytes

A fragment of 175 bp containing the 201 codon of GNAS exon 8 was amplified by PCR from blood tissue of all patients as previously described [13]. As direct sequencing is not a sensitive technique to detect mutant alleles present at low frequency, amplicons were subcloned into TOPO TA Cloning kit™ (Invitrogen, San Diego, Calif.) according to the manufacturer's instructions. The resulting construct was used to transform *Escherichia coli* strain Top Ten™ (Invitrogen, San Diego, Calif.). Cells were grown on medium selective for only those bacteria transformed by a plasmid containing an insert. Sequencing of 150 positive clones was performed for each patient's sample with BigDye™ terminator kit (Applied Biosystems, Foster City, CA) in an ABI PRISM 310 automatic sequencer (Perkin Elmer Cetus).

Results

Analysis of p.R201C and p.R201H mutations by real time PCR genotyping failure to clearly demonstrate the presence of any correspondent variant allele in blood and/or skin or adrenal samples of 31 MAS patients, with exception of the bone patient's sample used as p.R201H positive control (Figure 1a and b). Using cloning and sequence of amplicon from blood, we revealed that 5/150 clones contained the p.R201H in the same patient with the p.R201H in bone (Figure 2). Mutant clones could not be detected in any other patients.

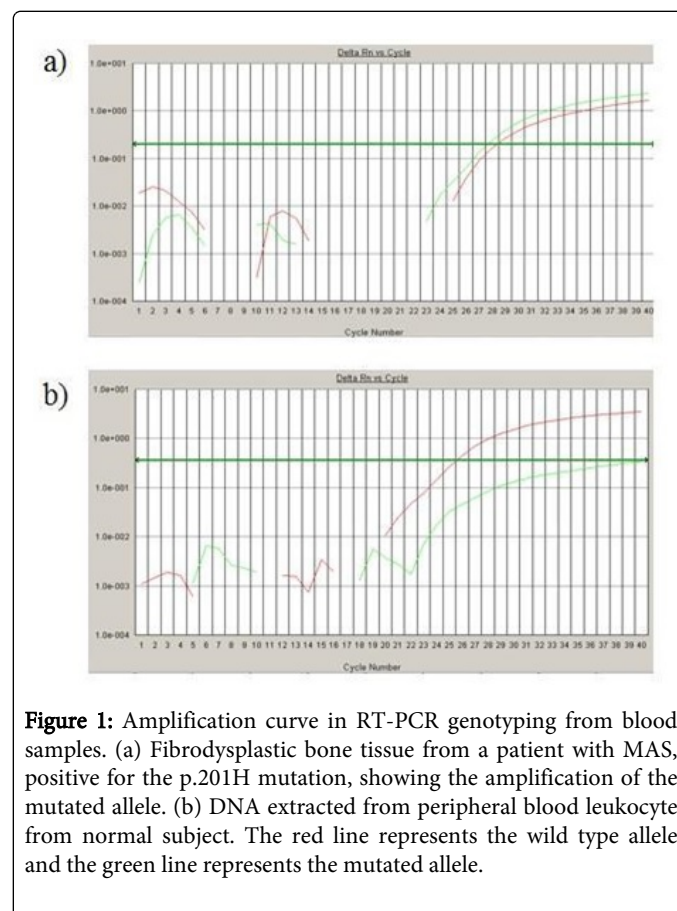


Figure 1: Amplification curve in RT-PCR genotyping from blood samples. (a) Fibrodysplastic bone tissue from a patient with MAS, positive for the p.201H mutation, showing the amplification of the mutated allele. (b) DNA extracted from peripheral blood leukocyte from normal subject. The red line represents the wild type allele and the green line represents the mutated allele.

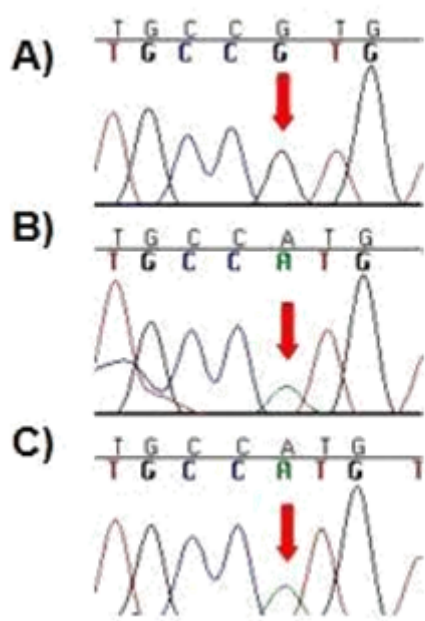


Figure 2: Electropherogram of the PCR product of GNAS. A) Peripheral blood leukocyte from a normal subject showing the amplification of wild allele only (arrow). B) Peripheral blood leukocyte from a patient with MAS, which the fibrodysplastic bone tissue was used as a positive control for the p.R201H mutation, showing the amplification of the mutated allele (arrow). C) Fibrodysplastic bone tissue from a patient with MAS, control for the p.201H mutation, showing the amplification of the mutated allele (arrow).

Discussion

We employed for the first time real time PCR genotyping to investigate GNAS mutation in MAS patients. Using this technique we detected a p.R201H mutation, previously identified by DGGE, in a bone sample of a patient with bone fibrodysplasia and precocious puberty (ref). However, the p.R201H mutations could not be detected in hematopoietic tissue of this patient using this same methodology. Then, we cloned and sequenced the amplicon of GNAS exon 1 fragment and confirmed the presence of the p.R201H in blood sample in 5/150 clones. The molecular diagnose of MAS is therefore highly dependent on the sensitivity of the technique used and also the tissue analyzed. Several techniques have been utilized, such as peptide nucleic acids (PNA) probes, restriction enzyme with nested PCR and direct sequencing by denaturing gradient gel electrophoresis (DGGE) [9,10,14]. The first techniques used to detect gsp mutations were described by Weinstein [5] and involved the amplification of the DNA for further analyzes for mutations by denaturing gradient gel electrophoresis and allele-specific oligonucleotides hybridization. The mutations were present in affected tissues obtained from patients with MAS. After that report, direct sequencing by DGGE was used in several studies, but requires several steps which can be inconvenient and make the technique slow [10,11]. Restriction enzyme with nested PCR method has also been used in several studies, but presented a high

risk of contamination inherent to nested PCR and compared with PNA, showed a lower sensitivity [10]. A study provided by Lietman and col showed that PNA-clamping provided an enhancement over standard polymerase chain reaction as well as a simple alternative to nested polymerase chain reaction 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 [14]. Schwindinger end colleagues, [6] using semiquantitative analysis, revealed that the mutant allele represented less than half of the alleles in this patient's leukocytes and that the exon 8 mutation was not detected in DNA from Epstein-Barr virus-transformed lymphoblasts, suggesting that the mutation was not present in all blood cells. They concluded that normal techniques could fail to reveal the Gsa mutation in DNA prepared, due to the very low number of copies of the mutant Ga mRNA. Sakamoto [15] used PCR-RFLP and direct sequencing analysis to detect the occurrence of GSa mutations at the Arg201 codon in fibrous dysplasias and osteofibrous dysplasias using formalin-fixed, paraffin embedded decalcified tissue. They couldn't identify the mutation and hypothesized that there were simply not enough mutant cells present to be detected by this method. The same result was described by Peleg and col [16]. They assume that the failure to identify the mutation in the DNA extracted from peripheral blood lymphocytes was due to the very low quantity of mutated DNA in these cells. Among all these techniques, only the PNA appears to be sensitive as was shown in several studies [9,14,17], although the real detection capacity was shown to be different in several studies, being able to detect from a range of 46 % up to 85 % of patients analyzed for the gsp mutations.

In conclusion, the mutation detection strategy described in the present study proved to be efficient for the molecular diagnosis of MAS in affected patient's tissues. Advantages of this technique are rapidity, accuracy, it is generally easy to perform and could be used routinely. Nevertheless, optimization of GNAS detection mutation in blood is still necessary to considerer the use of genotyping real time PCR to earlier diagnosis of non-classical forms of MAS.

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