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

Mariani BMP1,2, Trarbach EB2, Toledo RA3, Lerario AM1, Latronico AC3, Mendonca BB3 and Fragoso MCBV1

1Unidade de Suprarenal, Laboratorio de Hormonios e Genetica Molecular LIM42, Brazil
2Laboratorio de Endocrinologia Molecular e Celular/LIM2, Brazil
3Disciplina de Endocrinologia e Metabolologia do Hospital das Clinicas Facultade de Medicina da Universidade de Sao Paulo, Sao Paulo, Brazil

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

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.

Patients and Methods

Experimental subjects

The study population consisted of 31 patients with MAS followed at Hospital das Clinicas, 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      | Reverse     | Reporter dye     | FAN – mutated allele | VIC – wild type allele |
|---------------|--------------|-------------|------------------|----------------------|------------------------|
|               | CTTTG GTGAG ATCCATTGACCTCAA | AAT CTTTGAG ACCAAGTCCAGGTG | Probe designed for the detection of the p.R201H | TCG TGG CAC ATG TCC | TCG CTTG CCG GTG TCC |

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 kitTM (Invitrogen, San Diego, Calif.) according to the manufacturer’s instructions. The resulting construct was used to transform Escherichia coli strain Top TenTM (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 BigDyeTM 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) Fibrodsplastic 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.
risk of contamination inherent to nested PCR and compared with PNA, showed a lower sensitivity [10]. A study provided by Liemtan 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 consider the use of genotyping real time PCR to earlier diagnosis of non-classical forms of MAS.

Acknowledgement

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico- CNPq 830621/1999-7.

References

1. Albright FBA, Hampton AO, Smith P (1937) Syndrome characterized by osteitis fibrosa disseminata, areas, of pigmentation, and endocrine dysfunction, with precocious puberty in females: report of 5 cases. N Engl J Med 216: 727-746.
2. Grant DB, Martinez L (1983) The McCune-Albright syndrome without typical skin pigmentation. Acta Paediatr Scand 72: 477-478.
3. Rieth KG, Comite F, Shawker TH, Cutler GB Jr (1984) Pituitary and ovarian abnormalities demonstrated by CT and ultrasound in children with features of the McCune-Albright syndrome. Radiology 153: 389-393.
4. Danon M, Crawford JD (1987) The McCune-Albright syndrome. Ergeb Inn Med Kinderheilkd 55: 81-115.
5. Weinstein LS, Shenker A, Geiman PV, Merino MJ, Friedman E, et al. (1991) Activating mutations of the stimulatory G protein in the McCune-Albright syndrome. N Engl J Med 325: 1688-1695.
6. Schwindinger WF, Franchonato CA, Levine MA (1992) Identification of a mutation in the gene encoding the alpha subunit of the stimulatory G...
1. Kozasa T, Itoh H, Tsukamoto T, Kaziro Y (1988) Isolation and characterization of the human Gs alpha gene. Proc Natl Acad Sci U S A 85: 2081-2085.

2. Diaz A, Danon M, Crawford J (2007) McCune-Albright syndrome and disorders due to activating mutations of GNAS1. J Pediatr Endocrinol Metab 20: 853-860.

3. Idowu BD, Al-Adnani M, O'Donnell P, Yu L, Odell E, et al. (2007) A sensitive mutation-specific screening technique for GNAS1 mutations in cases of fibrous dysplasia: the first report of a codon 227 mutation in bone. Histopathology 50: 691-704.

4. Kalfa N, Philibert P, Audran F, Ecochard A, Hannon T, et al. (2006) Searching for somatic mutations in McCune-Albright syndrome: a comparative study of the peptidic nucleic acid versus the nested PCR method based on 148 DNA samples. Eur J Endocrinol 155: 839-843.

5. Fragoso MC, Lando VS, Latronico AC, Frazzatto ET, Russell AJ, et al. (2002) Detection of gsp somatic mutation through direct sequencing of heteroduplex alleles disclosed by denaturing gradient gel electrophoresis. Med Sci Monit 8: BR15-18.

6. Zuber TJ, Punch Biopsy of the Skin. Office Procedures, (Saginaw Cooperative Hospital, Saginaw, Michigan): 1161-64.

7. Happ GM, Aquilla E, Martick M, Yuncker C, Wojciechowski J, et al. (1999) DLA-DRB1 histocompatibility genotyping using RT-nested PCR and cycle sequencing. Vet Immunol Immunopathol 69: 93-100.

8. Lietman SA, Ding C, Levine MA (2005) 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. J Bone Joint Surg Am 87: 2489-2494.

9. Sakamoto A (2000) A comparative study of fibrous dysplasia and osteofibrous dysplasia with regard to Gsalpha mutation at the Arg201 codon: polymerase chain reaction-restriction fragment length polymorphism analysis of paraffin-embedded tissues. J Mol Diagn, 2: 67-72.

10. Peleg R, Luba A, Eliakim A, Israeli-Shani L, Manor E, et al. (2009) McCune-Albright syndrome in a discordant monozygotic twin. Isr Med Assoc J 11: 343-347.

11. Riminucci M, Fisher LW, Majolagbe A, Corsi A, Lala R, et al. (1999) A novel GNAS1 mutation, R201G, in McCune-albright syndrome. J Bone Miner Res 14: 1987-1989.