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Germline mosaicism in Coffin-Lowry syndrome

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We have identified a Coffin-Lowry syndrome pedigree where the disorder is associated with a novel splice site mutation in the *RSK2* gene, leading to in-phase skipping of exon 5. Western blot analysis, using an antibody directed against the C-terminus of *RSK2*, failed to reveal *RSK2* in this patient, suggesting strongly that the resulting internally deleted protein is unstable. The mutation was present in the DNA of one affected son and one manifesting daughter but was absent in two asymptomatic daughters, who carry the at-risk haplotype, and in the mother's somatic cell (lymphocyte) DNA. The results are consistent with the mutation arising as a postzygotic event in the mother, who therefore is a germinal mosaic. The application of linked markers to identify the disease allele for conventional genetic counselling would have been misleading in this family. This observation again highlights the importance of precise identification of the disease-causing mutation.

Keywords: Coffin-Lowry syndrome; RSK2 mutation; germline mosaicism

Introduction

The Coffin-Lowry syndrome (CLS) is a rare X-linked disorder characterised, in male patients, by severe mental retardation, typical facial changes, proximally puffy digits, and progressive skeletal deformations.[1–4] Manifestations in female carriers are highly variable, but generally the signs and symptoms are much less severe than in males, with often normal or near-normal intelligence. Heterozygote detection is possible in most but not all carrier females.[4,5] Although the actual incidence of this disorder is unknown, it seems to occur in about equal and not extremely low frequency in various ethnic groups (see references in Haspeslagh *et al*[5]).

Genetic mapping studies placed the CLS locus to a region of approximately 3 cm in Xp22.2.[6–9] Within this interval we recently identified the gene mutated in CLS patients: it encodes RSK2, a serine-threonine protein kinase[10] involved in cell proliferation and differentiation.[11]

To search for *RSK2* mutations in CLS patients, the genomic organization of the *RSK2* gene has been determined: it contains 22 small coding exons (SJ, submitted). Up to now, 10 different RSK2 mutations have been found in CLS families.[10,12] All are unique and are spread throughout the gene without preferential localisation.

In this report, a novel splice site mutation in the *RSK2* gene has been identified in a CLS patient. Furthermore, we provide the first molecular and

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genetic evidence for the occurrence of germline mosaicism in Coffin-Lowry syndrome.

Patients and Methods

Family

The propositus, a male, is the first child of his mother who was born when she was 23 1/2 years old. The mother is the oldest of seven sibs; she was born to a 26 year-old father and a 21 year-old mother and has four healthy brothers and two healthy sisters, all with normal offspring. The proband was born 10 days over expected term weighing 2220 g (< 10th percentile). When the child was only 3 weeks old, the mother consulted a paediatric neurologist who suspected an abnormal neurologic status and initiated physiotherapy. At this point, bilateral inguinal hernias and unilateral hydrocele were operated. Breast feeding was without problems. Psychomotor and mental development were delayed from the beginning. On one of several hospitalisations, at age 6 years, Coffin-Lowry syndrome was clinically diagnosed. Severe scoliosis and coarsening of the face developed, the former necessitating operation at age 16 years. At the last clinical examination at age 26 1/2 years, head circumference was 56.5 cm (50th–75th percentile); height could not be reliably measured but was estimated to be below 1.50 m. He showed the following symptoms: narrow bitemporal diameter and forehead, coarse facial features, prominent brows and periorbital fullness, hypertelorism (inner canthal distance 3.5 cm, about 90th percentile), downslanting palpebral fissures, a broad nose with thick alae and septum, permanently open mouth with thick and protruding lips, dental crowding, prominent lateral palatine ridges, large and protruding ears (ear length 7.5 cm bilaterally, about 90th percentile), pectus excavatum, severe scoliosis, a narrow pelvis, hypoplastic male genitalia, flexion contractures of all larger joints of arms and legs, hand length 19.5 cm (75th percentile), bilateral transverse palmar creases, tapering fingers with hypermobility in all joints, subluxable metacarpo-phalangeal joints of thumbs, flat feet with a foot length of 23 cm (< 3rd percentile), cutis marmorata. There was severe to profound mental deficiency. Subsequent to the birth of her first child — the propositus — the mother had three daughters by another partner. The first and third are normal in growth and development and are now aged 23 and 15 years, respectively. The second daughter was born when mother and father both were 33 years old. Her birthweight at 39 2/7 weeks of gestation was 3100 g (50th percentile). Developmental delay, although less pronounced than in her half-brother, was first suspected at 8–10 months. She could sit unsupported at 12 months and in an upright position at 18 months. Length/height and head circumference have always been within normal limits. She underwent special education and now, at age 17 years, attends a day school for the mentally handicapped. At the last examination at age 17 1/2 years, she showed a mildly dysmorphic longish face with periorbital fullness (but less marked than in her half-brother), downslanting palpebral fissures, a narrow nose, small and crowded teeth, mild pectus excavatum and hypertrichosis. Muscle tone is decreased.

SSCP analysis

PCR reaction of exon 5 and flanking intron boundaries was carried out in a reaction volume of 25 μl, containing 100 ng DNA. 1.5 mM $MgCl_2$, 5U Taq polymerase (Perkin Elmer, Roche Molecular Systems, Inc, Branchburg, New Jersey, USA), 1 pmol (3 μCi) of ^{32}P dCTP and the following primers: 5'-AGTGGATATTACAATGTAGCA-3' and 5'GGGAGACGGCTCATACTTA-3'. A sequence of about 140 bp was amplified using the PTC-100 Programmable Thermal Controller (MJ Research) at 94°C for 5 min followed by 30 cycles of 94°C for 10 s, 55°C for 10 s, 72°C for 10 s, and a final elongation step at 72°C for 5 min. After PCR, aliquots of 4 μl were denatured for 5 min at 90°C and loaded on a 10% glycerol, 6% polyacrylamide gel (acryl/bisacryl, 49:1). Electrophoresis was run at 4W for 10–12 h at 4°C. The gel was dried and exposed overnight. The PCR product from the proband was sequenced directly on an automated DNA sequencer.

RT-PCR

Total RNA was prepared from a lymphoblastoid cell line, established from the proband, as previously described.[10] RT-PCR was performed on 1 μg of total RNA as previously described,[10] using the following primers: 5'-TCTATAAATTATTCCAGGCT (reverse transcription), 5'-ATTATTCCCAGGCTATGTAG, and 5'-TTAGTTAAAAAAATCTCAGGCTCTGATGC (subsequent PCR amplification). The PCR amplified cDNA was sequenced on an automated DNA sequencer.

Western analysis

Total protein extractions from cell lines and western-blot analysis were performed as previously described.[10] Prior to western-blot analysis, an immunoprecipitation step was introduced in order to increase the concentration of RSK2 protein. Immunoprecipitation was carried out with the C-19 goat polyclonal antibody specific for RSK2 (which, however, shows cross-reaction with RSK3) (Santa Cruz Biotechnology, Santa Cruz, California, USA), and after blotting, membranes were incubated with the same antibody.

Haplotype analysis

Markers DXS 443, DXS 999, DXS1229, DXS 365 and DXS1683 were analysed as previously reported.[13,14]

Results

Mutation detection of genomic DNA from the proband was performed by PCR amplification of 6 exons (exons 5, 6, 7, 13, 16, 20), for which flanking intron sequences were known. Single strand conformation polymorphism (SSCP), analysis of the PCR product containing exon 5 showed mobility shift in the proband, compared with control individuals. Direct sequencing of the PCR product revealed a G to A transition of the splice acceptor site (position –1) immediately upstream of exon 5. Subsequent direct sequencing of an RT-PCR product from the proband, of which a lymphoblastoid cell line was available, revealed in-phase skipping of exon 5 (Figure 1). Western blot analysis, using the C-19 antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) directed against the C-terminus of *RSK2*,

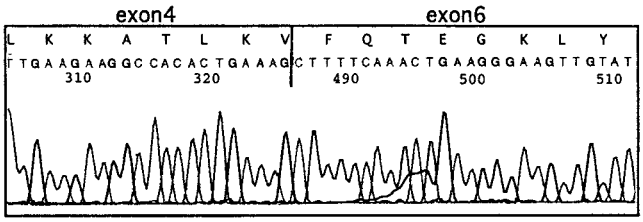

Figure 1 *Sequence analysis of an RT-PCR product from the proband, showing skipping of exon 5 in the RSK2 cDNA. RT-PCR and direct nucleotide sequence determination were performed as described in the method section. Nucleotide numbering of the RSK2 cDNA sequence is as previously reported in Bjorbaek et al.*[21]

failed to reveal *RSK2* in the proband, suggesting strongly that the resulting internally deleted protein is unstable (Figure 2). The presence of this mutation was then tested in the leucocytes of the mother and the three half sisters of the proband by SSCP analysis. Figure 3 shows the SSCP analysis of exon 5 in the whole family. One half sister was found heterozygous for the band shift seen in the proband and a normal band pattern, whilst the mother and the two other half sisters were homozygous for the normal pattern. Family members were genotyped for five microsatellite polymorphisms, closely linked to the CLS locus, (DXS443, DXS999, DXS1229, DXS365 and DXS1683) as previously reported.[13,14] The results of haplotype analysis are shown in Figure 4: microsatellites DXS 1683 and DXS 999, flanking the CLS locus proximally and distally respectively, were informative. The patient and his three half sisters all had inherited the same haplotype from their mother.

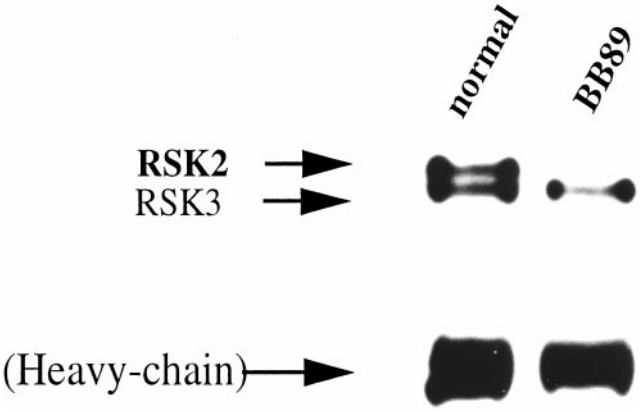

Figure 2 *Western analysis of immunoprecipitated RSK2 proteins (740 amino acids) from lymphoblastoid cell lines of a normal control individual and the proband (BB89). RSK3 protein (733 amino acids) is also immunoprecipitated and detected by the C-19 anti-RSK2 antibody (Santa Cruz Biotechnology). The immunoglobin heavy chain (immunoprecipitation) is revealed, while the light chain has migrated out of the gel.*

Discussion

Here we report on a family with Coffin-Lowry syndrome in which one son is affected and one daughter is partially expressing, whilst two other daughters and the mother are asymptomatic. A G-to-C transversion was

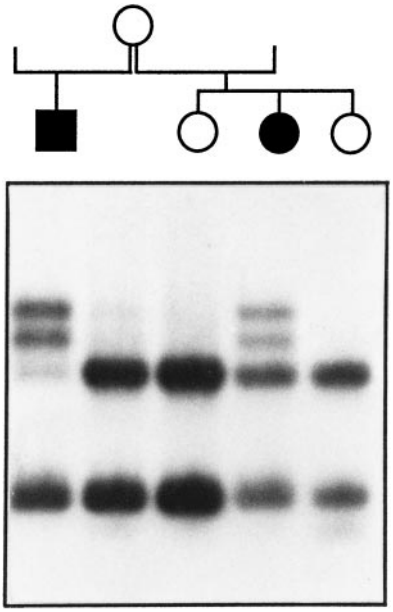

Figure 3 *SSCP analysis of exon 5 and flanking intron boundaries. The proband (close square) shows a band shift caused by a splice site mutation (326-1 G- > C). One half-sister (close circle) has both a shifted and a normal band, confirming that she is a CLS carrier, whilst the other two half-sisters (open circles) and their mother exhibit a normal band pattern.*

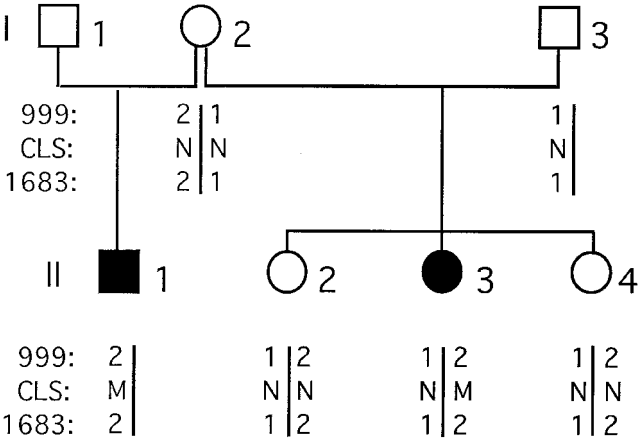

Figure 4 *Pedigree showing haplotypes for CLS flanking markers.*

demonstrated in the splice acceptor site of intron 4 of the *RSK2* gene in the proband, leading to in-phase skipping of exon 5. However, western blot analysis, using an antibody directed against the C-terminus of *RSK2*, failed to reveal the RSK2 protein in the proband (Figure 2). The internally deleted RSK2 protein is thus presumably unstable, resulting in a null allele. The early evidence in this family was consistent with the mother being a non-manifesting carrier who had passed the disease allele to her son and at least to the manifesting daughter. One half-sister, designated as a carrier based on a partial expression of the disease, also did carry the mutation. This change was present, however, neither in the other two half-sisters of the proband, who inherited the at risk haplotype, nor in the peripheral blood lymphocytes of the mother, thus providing unequivocal evidence of maternal germline mosaicism. Furthermore, SSCP analysis of the mother's genomic DNA, in addition to the strong wild type bands, revealed repetitively very faint bands that may correspond to the presence of a very small proportion (< 1%) of mutated alleles (Figure 1). Thus, there is faint suggestion of somatic mosaicism. However, a slight contamination of the mother's genomic DNA, with mutated PCR products or another genomic DNA, could not be excluded, and no new sample was available.

Our results emphasise the importance of detection of the causative mutation for carrier detection and prenatal diagnosis. The application of linked markers to identify the disease allele for conventional genetic counselling would have been erroneous in this family. This is of particular importance to the two daughters, who have inherited the at-risk haplotype but do not carry the mutation.

About 70% of patients with clinical features suggestive of Coffin-Lowry syndrome in our series of 90 families are sporadic cases (ie no family history of the disease). As stated above, to date a mutation in the *RSK2* gene has only been demonstrated in ten families, including seven sporadic cases.[10,12] In four of the latter families the mutation arose *de novo*.[15] Thus, the high proportion of sporadic cases might indeed reflect a high rate of new mutations in the *RSK2* gene. However, studies of additional families with identified mutations are worthwhile to confirm this very preliminary result. Germline mosaicism, with or without somatic mosaicism, has been reported for many X-linked disorders, including Duchenne muscular dystrophy (DMD), haemophilia A, severe combined immunodeficiency (SCID) and X-linked hydrocephalus.[16–20] The incidence of germline mosaicism has been reported to be as high as 14% for the at-risk haplotype in new cases of DMD. Thus, possible germline mosaicism for RSK2 mutations has important counselling implications for CLS families, especially those with isolated cases. The absence of a mutation in the mother of a sporadic Coffin-Lowry patient does not rule out a recurrence risk for future pregnancies, and prenatal diagnosis should be offered. In addition, carrier detection should be performed on the sisters of male patients in such families. Further studies are needed to determine the incidence of gonadal mosaicism in CLS.

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