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Evidence of somatic mosaicism for a *MECP2* mutation in females with Rett syndrome: diagnostic implications

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EDITOR—Rett syndrome (RTT) (MIM 312750) is an X linked dominant neurodevelopmental disorder that occurs almost exclusively in females. Affected girls are considered to have a normal perinatal period followed by a period of regression, loss of acquired purposeful manual and speech skills, hand wringing, gait disturbance, and growth retardation.[1 2]

A gene for RTT has been identified in the Xq28 region which encodes the methyl-CpG

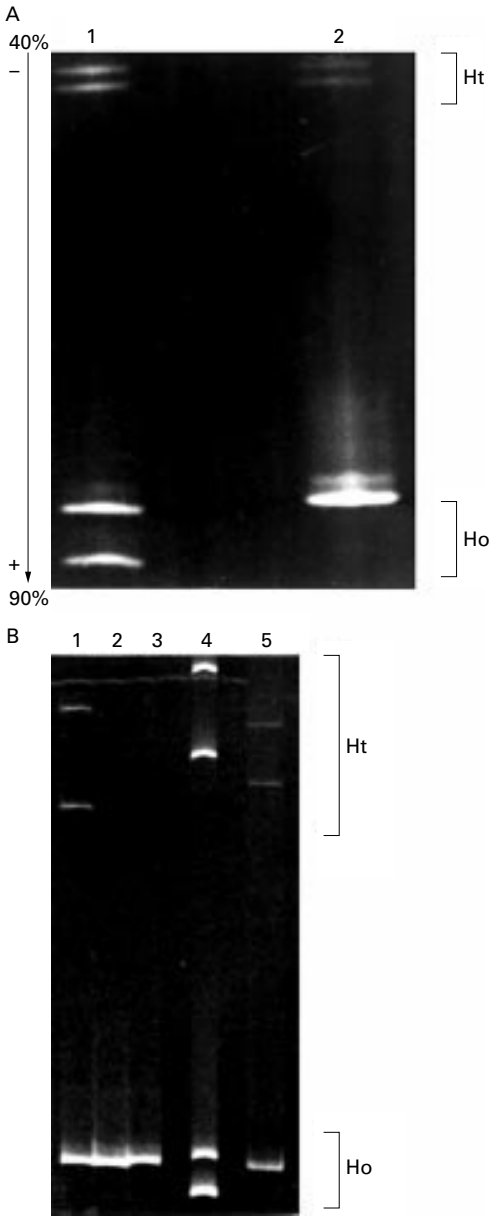

Figure 1 Detection of the two somatic mutations by heteroduplex analysis. (A) DGGE results for case 1. DNA extracted from a lymphoblastoid cell line (lane 1) and a fresh blood sample (lane 2). The homoduplex band corresponding to the deleted allele is missing in DNA extracted from lymphocytes. Ht, heteroduplex; Ho, homoduplex; 40%-90% formamide gradient. (B) CSGE results for case 1 (lanes 1-3) and case 2 (lane 5). Case 1: the somatic mutation is shown on DNA extracted from a fresh blood sample (lane 1); the father (lane 2) and the mother (lane 3) do not carry the 26 bp deletion. Case 2: a somatic deletion was detected on DNA extracted from lymphocytes as indicated by the absence of the deleted homoduplex band (lane 5). Lane 4 depicts a CSGE pattern of a 31 bp deletion localised in the deletion prone region of the MECP2 gene; the two homoduplex bands are of equal intensity.

binding protein 2 (MeCP2) involved in transcriptional silencing.[3 4] This disorder most frequently occurs sporadically and results from a de novo mutation, although a few familial cases have been reported. Many studies[5–16] have shown that the *MECP2* gene is mutated in approximately 80% of patients with classical RTT and the *MECP2* mutation spectrum includes missense, nonsense, and frameshift mutations, as well as larger rearrangements like deletions encompassing a few hundred bp.[16] The failure to detect *MECP2* mutations in the remaining 20% may indicate the presence of mutations in unexplored regions of the *MECP2* gene, such as regulatory elements or non-coding regions, notably in the new first exon[17] or in an additional RTT locus.

Here, we report for the first time mosaicism for a somatic *MECP2* mutation found in two unrelated females affected with RTT. These two girls were diagnosed according to the international criteria of the Rett Syndrome Diagnostic Criteria Work Group.[18]

Case reports

The first patient (case 1) is 13 years old. She suffers from classical Rett syndrome with 7/9 of the necessary criteria, 4/8 of the supportive criteria, and none of the exclusion criteria.[18] More specifically, she had a normal neonatal period and head circumference at birth and a phase of social withdrawal at the age of 12 months when she lost purposeful hand skills and developed stereotypic hand movements, ataxia, and apraxia. She suffered from breathing dysfunction and peripheral vasomotor disturbances. She had severely impaired development but acquired independent walking at the age of 24 months. However, she did not acquire microcephaly or develop epilepsy.

The second patient (case 2) was reported as an atypical case of RTT without any period of regression. Both mental and motor development were very slow. At the age of 4 years, she had acquired microcephaly (−2 SD) and had very limited ambulation, but her hand use was correct without hand wringing movements. She developed epilepsy and progressive scoliosis. She is a placid girl without useful speech but she communicates well by eye movements.

Methods and results

For case 1, an initial study on DNA extracted from a lymphoblastoid cell line by denaturing gradient gel electrophoresis (DGGE) and sequencing showed that she carried a 26 bp deletion starting at position 1165. To confirm this mutation, DNA was extracted from a fresh blood sample and the deletion was assessed by direct sequencing. Surprisingly and despite a careful examination of the sequence, we did not find the 26 bp deletion with DNA extracted from leucocytes. This sample was reanalysed by DGGE and heteroduplexes were detected while the homoduplex corresponding to the deleted band was absent (fig 1A). We confirmed this result by conformation sensitive gel electrophoresis (CSGE) analysis, which showed the heteroduplexes but not the mutant homoduplex (fig 1B). The results obtained from peripheral blood lymphocytes suggested mosaicism for a somatic mutation.

In order to determine the level of mosaicism, we used a semiquantitative approach based on fluorescent PCR. The *MECP2* gene exon 3 portion containing the deletion was PCR amplified, the reverse primer being conjugated

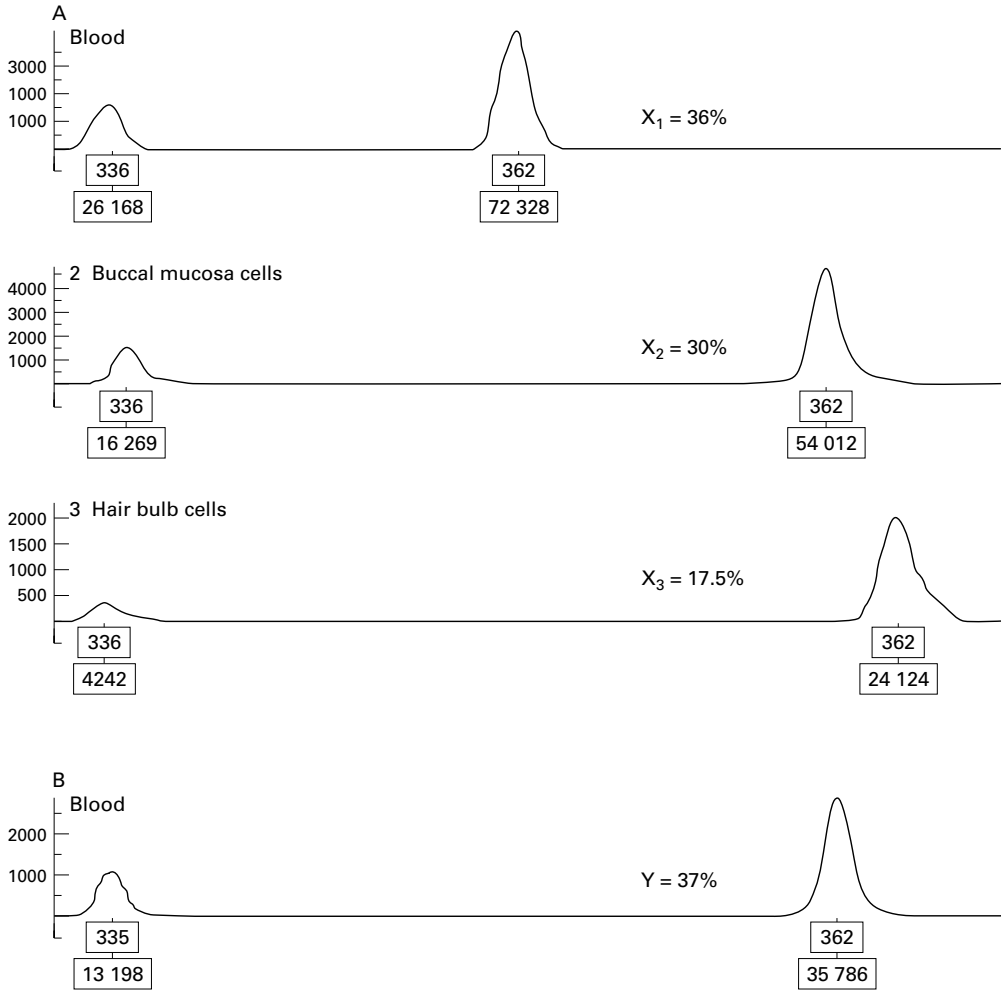

Figure 2 Semiquantitative fluorescent PCR of the somatic mosaicism rate. (A) Case 1. Genotyper traces of the fluorescent PCR products obtained with three different tissues, blood (1), buccal mucosa cells (2), and hair bulb cells (3), shown with the three respective ratios of peak areas (X_1, X_2, X_3). For each peak, the fragment size in bp and the peak area calculated by Genescan is indicated. We assumed that the mosaicism rate could be estimated by calculating the ratio between the deleted and the normal peak areas. (B) Case 2. Genotyper trace of a fluorescent PCR product obtained from blood with the ratio of peak areas (Y).

to 6-FAM (6-carboxy-fluorescein). PCR products were analysed on an ABI 310 sequencer and peak areas were generated by ABI Genescan and Genotyper software. The ratio between the deleted and normal peak areas showed that only 36% of lymphocytes harboured the deletion, that is, 18% of X chromosomes bore the 26 bp deletion (fig 2A). This semiquantitative approach confirms that case 1 does have somatic mosaicism for the *MECP2* deletion. The relatively low level of somatic mosaicism could explain the normal sequencing result. Thus, mosaicism was quantified in different tissues. DNA was extracted from buccal mucosa cells[19] and hair bulb cells. [20] The level of mosaicism was about the same in buccal mucosa cells (30%) as in lymphocytes, but lower in hair bulbs cells (17.5%) (fig 2A).

Discussion

On the basis of these results, we hypothesised that some patients with RTT may in fact carry a somatic mutation. Small deletions (from 7 to 170 bp) within the region between bp 1096 and 1165 of the *MECP2* gene have been recurrently identified.[5 7 9 10 12 15 16] They do not affect the two functional domains but result in the loss of one fifth of the protein. Interestingly, it has been shown that the deletion of the carboxy-terminal 63 amino acids of the MeCP2 protein impairs binding with the nucleosomal DNA during the transcription regulation process.[21] These recurrent deletions may be the result of palindromic and quasipalindromic sequences within this region, which are believed to form secondary structures that render the region vulnerable to deletions. Therefore, using our fluorescent PCR approach, we reanalysed the 3' region of the *MECP2* gene, between bp 1096 and 1165, in a cohort of 29 patients diagnosed as typical or atypical RTT; for these patients, we failed to detect any mutation using a bidirectional sequencing strategy of the entire *MECP2* coding region. A second somatic mosaicism for a 27 bp deletion was identified in peripheral blood lymphocytes from case 2 with atypical RTT; the mosaicism rate was quantified with our fluorescent approach to be about 37% (fig 2B). We confirmed this result by CSGE analysis (fig 1B).

In both cases, numerical aberrations of the X chromosomes as a cause for the uncommon fluorescent PCR patterns were excluded by the presence of a normal 46,XX karyotype.

These two patients show a similar deletion with an equivalent mosaicism rate in blood, but a distinct clinical presentation. X inactivation study on proband 1 with typical Rett syndrome showed a random pattern of inactivation in the peripheral blood. Although the results have to be extrapolated from the peripheral blood cells, it would suggest that in the brain the majority of mutated X chromosomes may remain active in the girl with classical Rett syndrome. Our results illustrate clearly once again the difficulty in establishing a correlation between genotype and phenotype in RTT.

Recently, a boy with a mosaic mutation has been described.[22] To our knowledge, we show for the first time that somatic mosaicism for *MECP2* mutation in girls is not infrequent (two somatic mutations on 102 putative RTT cases studied) and may cause different phenotypes. These clinical and molecular findings suggest that multiple forms of mosaicism (X inactivation mosaicism and somatic mosaicism) may be present in a single patient with RTT. Mosaicism has been documented for chromosomal abnormalities, mitochondrial mutations, triplet repeats,[23] and in a growing number of dominant and recessive X linked gene disorders, such as Duchenne muscular dystrophy,[24] haemophilia B,[25] Conradi-Hünermann-Happle syndrome,[26] and double cortex/lissencephaly syndrome.[27] Because a proportion of cells carry the mutation not only in blood but also in tissues deriving from other cell lineages, it must be assumed that the mutation occurred very early during embryogenesis.

Finally, the detection of mosaic mutation depends mainly on the method used for the identification of mutations within the *MECP2* gene. Nowadays, the method of choice for identifying deleterious mutations relies on direct DNA sequencing. The ability of this method to detect mosaic mutations is poor, which is particularly true when the mosaicism rate is low. Our findings underline the need for at least two complementary approaches, such as methods based on heteroduplex analysis and sequencing, for an efficient screening of the *MECP2* gene.

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Temperature sensitive acyl-CoA oxidase import in group A peroxisome biogenesis disorders

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EDITOR—Peroxisome biogenesis disorders (PBDs) are lethal genetic diseases characterised by a number of peroxisomal metabolic abnormalities, including the oxidation of very long chain fatty acids (VLCFAs), biosynthesis of bile acids and plasmalogen, and detoxification of H_2O_2. Peroxisomal matrix proteins are synthesised on free polyribosomes and directed to the organelle by *cis* acting peroxisome targeting signals (PTSs). PTS1 is a C-terminal tripeptide Ser-Lys-Leu (SKL) sequence and later the consensus sequence was broadened to (S/A/C/K/N)-(K/R/H/Q/N/S)-L, based on subsequent studies. Acyl-CoA oxidase (AOX) has SKL and D bifunctional protein has AKL.[1–4] PTS2 is an N-terminal cleavable peptide (-R/KLX5Q/HL) that resides in peroxisomal 3-ketoacyl CoA thiolase (PT), alkyldihydroxyacetonephosphate synthase, and phytanoyl-CoA hydroxylase.[5–9] PBDs are genetically classified into at least 12 complementation groups (CGs) and each CG contains various clinical phenotypes, for example, Zellweger syndrome (ZS), neonatal adrenoleucodystrophy (NALD), and infantile Refsum disease (IRD).[10 11] ZS patients have severe neurological defects, liver dysfunction, and renal cysts and die before 1 year of age. NALD patients have symptoms similar to ZS patients, but they survive a little longer, and IRD patients show milder abnormalities in the central nervous system and survive even longer. We identified the restoration of peroxisome biogenesis in a temperature sensitive (TS) manner in fibroblasts from milder forms of PBDs, that is, all IRD patients and some NALD patients belonging to groups CG-A (CG8), CG-C (CG4), CG-E (CG1), CG-F (CG10), CG-H, and CG6.[12–15] In these cells, peroxisomes were formed at 30°C and biochemical activities of peroxisomes, including the oxidation of VLCFAs and dihydroxyacetonephosphate acyltransferase (DHAP-AT), and the import of peroxisomal enzymes, were also restored.[16] However, virtually no peroxisomes were formed in ZS cells at 30°C and import of peroxisomal enzymes did not improve.[16] Here, we elucidate temperature dependent import and processing of AOX at 30°C which is unique to fibroblasts from ZS patients belonging to CG-A. Correlation between the import of peroxisomal enzymes and biochemical functions of peroxisomes is also discussed.

Materials and methods

CELL LINES

Skin fibroblasts from the patients belonging to CG-A (CG8) including four with ZS (A-02, 06, 10, and 14), two with NALD (A-05 and 08), and one with IRD (A-04) were cultured at 37°C or 30°C in an atmosphere of 5% CO_2 in MEM supplemented with 10% fetal calf serum. A-02, A-06, and A-14 were Japanese babies diagnosed as ZS with typical dysmorphic features, who died at a few months of age. The clinical data of A-04 and A-08 have been previously reported, whereas those of A-05 and A-10 have not.[12] In addition, ZS fibroblasts belonging to CG-C (C-08), CG-E (E-14), and CG-F (F-01) were cultured under the same condition (the numbers and clinical data of these patients have been previously described[12]). All cell lines were classified by complementation analysis as previously described.[10 17 18]

IMMUNOFLUORESCENCE STUDY

For the detection of peroxisomes and the import of PTSs, cells were fixed after 72 hours' incubation at either 37°C or 30°C, permeabilised with 0.1% Triton X-100, and processed for indirect immunofluorescence staining.[19] The first antibodies we used were rabbit antibodies to human catalase, AOX, D bifunctional protein, and PT, and in double immunofluorescence rabbit anti-rat PMP70 antibody was used.

BIOCHEMICAL ASSAYS

Peroxisomal VLCFA oxidation in fibroblasts was assessed by the ratio of lignoceric acid (C24:0)/palmitic acid (C16:0) oxidation activity.[20] The activity of DHAP-AT, the first enzyme in the pathway leading to plasmalogen biosynthesis, was measured as described previously[21] using ^{14}C labelled DHAP as substrate. Continuous cell labelling with ^{35}S-methionine and immunoprecipitation of AOX with rabbit anti-human AOX antibody was performed as described previously.[19 22]

Results

IMMUNOFLUORESCENCE STUDY IN FIBROBLASTS FROM CG-A PATIENTS

The fibroblasts from PBD patients belonging to the CG-A were examined by immunofluorescence microscopy to determine the import of PTS1 and PTS2 containing peroxisomal matrix proteins. The immunoreactivity of these proteins in control cells showed the same punctate pattern as previous reports (data not shown).[23] The most striking result was that in the fibroblasts from ZS patients, the import of AOX was rescued apparently after incubation at 30°C, whereas it was severely reduced or absent at 37°C (fig 1C, D, table 1).