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SHORT REPORT

Parental origin of *de novo MECP2* mutations in Rett syndrome

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Rett syndrome (RTT) is a neurodevelopmental disorder occurring almost exclusively in females as sporadic cases. Recently, DNA mutations in the *MECP2* gene have been detected in approximately 70% of patients with RTT. To explain the sex-limited expression of RTT, it has been suggested that *de novo* X-linked mutations occur exclusively in male germ cells resulting therefore only in affected daughters. To test this hypothesis, we have analysed 19 families with RTT syndrome due to *MECP2* molecular defects. In seven informative families we have found by DHPLC a nucleotide variant which could be used to differentiate between the maternal and the paternal allele. In each subject investigated from these families, we have amplified specifically each allele and sequenced allele-specific PCR products to identify the allele bearing the mutation as well as the parental origin of each X chromosome. This approach allowed us to determine the parental origin of *de novo* mutations in all informative families. In five cases, the *de novo MECP2* mutations have a paternal origin and in the two other cases a maternal origin. In all transitions at CpG, the *de novo* mutation observed was of paternal origin. The high frequency of male germ-line transmission of the mutation (71% of RTT informative cases) is consistent with a predominant occurrence of the disease in females. *European Journal of Human Genetics* (2001) **9,** 231 – 236.

Keywords: *MECP2* gene; Rett syndrome; parental origin

Introduction

Rett syndrome (RTT) is generally considered as an X-linked dominant disorder with male lethality.[1] It affects approximately 1 in 10 000 to 15 000 females. The cause of RTT has been debated in the literature for more than a decade. Recently, the discovery that RTT is caused by mutations in the *MECP2* gene, located on Xq28, proved the developmental origin of the disease.[2] *MECP2* encodes the methyl-CpG-binding protein 2, a ubiquitous DNA-binding protein that is thought to act as a global transcriptional repressor. The protein's 84 amino acid methyl-binding domain binds 5-methyl cytosine residues in CpG dinucleotides. The 104-amino acid transcriptional repression domain interacts with the corepressor Sin3A to recruit histone deacetylases, which in turn deacetylate of core histones and transcriptional silencing.[3] An aberration in MeCP2 function could thus lead to dysregulation of a number of other genes.

Several recent reports found that approximately 70% of sporadic RTT patients have missense or truncated mutations. Consistent with the sporadic occurrence of RTT, most of these mutations occurred *de novo* at CpG mutation hotspots.[4,5] The majority of the single-nucleotide substitutions are C to T transitions at CpG sites (R106W, R133C, T158M, R306C, R168X, R255X) (71% of all identified *MECP2* mutations).[3,6] These sites are hypermutables. The proposed mechanism involves 5-methylation of cytosine by a methyltransferase and spontaneous deamination of 5-methylcyto-

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sine to thymine. CpG hypermutability implies that the site is methylated in the germline and thus is prone to deamination. Male germ cells have high levels of CpG methylation, and the X chromosome, in particular, is completely inactivated. Therefore, the *MECP2* gene is likely to be also methylated.

To explain the sex-limited expression of RTT, it has been suggested that the *de novo* X-linked mutations occurred exclusively in male germ cells and resulted in affected daughters.[7] Under such a hypothesis, the absence of affected males is explained by the fact that sons do not inherit their X chromosome from their fathers.

To test this hypothesis, we have analysed 19 families with RTT syndrome due to *de novo MECP2* molecular defects. All patients presented a previously identified mutation in the *MECP2* gene and had phenotypically normal parents, and in each case the correct paternity was proven. In seven informative families we found a nucleotide variant located in the *MECP2* gene which could be used to differentiate between the maternal and the paternal allele. In each subject investigated, we amplified specifically each allele and sequenced the allele-specific PCR products. Study of the segregation of the X chromosome bearing the variant and the pathogenic mutation allowed us to determine the germ-line origin.

Materials and methods

Subjects and DNA samples

We studied 19 sporadic patients, all of whom fulfilled the criteria for the diagnosis of Rett syndrome.[1] All the patients presented a mutation in the *MECP2* gene: four R270X; three R168X; three T158M; three R294X; one R255X; one 1156del17; one P302R; one X487C; one 677insA and one 1163del26. We prepared total genomic DNA from peripheral blood leukocytes cell lines using standard protocols.

Denaturing HPLC analysis

The search for polymorphisms in the *MECP2* gene was performed by denaturing high performance liquid chromatography (DHPLC) scanning on an automated HPLC instrument (Wave technology). The stationary phase consisted of 2 μm nonporous alkylated poly(styrene-divinylbenzene) particles packed into a 50×4.6-mm i.d. column (Transgenomic, Santa Clara, CA, USA). The mobile phase was 0.1 M triethylammonium acetate buffer at pH 7.0 containing 0.1 mM EDTA. The temperature required for successful resolution of heteroduplex molecules was determined empirically by injecting and running PCR products at increasing mobile phase temperatures, usually in 1–2°C increments starting from 50°C until a significant decrease in retention of approximately 1 min was observed. A total of 14 primer pairs listed in Table 1 were used for amplifying parts of the 5′ UTR, intron 1, intron 2 and 3′ UTR regions of the *MECP2* gene.

PCR amplification of specific alleles

For allele-specific amplifications, the technique requires two oligonucleotides primers identical in sequence except for the terminal 3′ nucleotides, one of which is complementary to the normal DNA sequence and the other to the changed nucleotide in the mutant DNA.[8,9] Under carefully controlled conditions a primer with its terminal 3′ nucleotide mismatched will not function properly and no amplification occurs from the wild type allele. In two cases, a single mismatched base was introduced three nucleotides from the 3′ end of both primers to enhance their specificity. Allele-specific primers used for each identified polymorphism are listed in Table 2. A control pair of primers was included in each assay. The control primers amplify a region of *IL-1RAPL* gene.[10] The size of the amplified control fragment (internal standard in the figures) is different from the fragments produced by the allele-specific primers. PCR reactions were performed using an automated 9700 DNA thermal cycler (Perkin Elmer) in a total of 50 μl containing 250 ng of genomic DNA, 2.5 mM $MgCl_2$, 0.25 μM of each primer, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 200 μM of each dNTP and 0.5 units of Taq DNA polymerase. Thirty-five cycles were performed with denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and elongation for 1 min at 72°C. PCR products were identified by agarose gel electrophoresis.

Sequence analysis

For each fragment that displayed a heteroduplex peak in at least one individual and for each allele-specific fragment, PCR products were purified on solid-phase columns using the Qiaquick PCR purification kit (Qiagen) then sequenced in both directions on an ABI 373 automated sequencer using the dye-terminator cycle sequencing reaction kit (Perkin-Elmer).

Results

To carry out polymorphism screening by DHPLC, we have designed appropriate primers to analyse the noncoding regions of the *MECP2* gene, and for each amplified fragment we determined the optimal conditions to detect single-base substitutions.[11,12] Investigation of 14 fragments covering the noncoding part of *MECP2* gene (three fragments in 5′ UTR, four in intron 1, three in intron 2, and four in the 3′ UTR region) identified in 11/19 RTT families (F1 to F19) the presence of six sequence variations (Table 3). One is located in 5′ UTR, two in intron 1, three in intron 2 and four in the 3′ UTR region (Figure 1). Two out of the six biallelic polymorphisms were heterozygous in 31% (6/19) of investigated cases.

In only seven RTT families (F2, 3, 7, 8, 9, 10 and 11), we can differentiate in the affected patient the maternal and the paternal allele by the presence of one (or more) nucleotide variant (Table 3). These RTT families present the following *MECP2* mutations: two R294X, one R270X, one R168X, one

Table 1 Primer sequence and amplification parameters for *MECP2* fragments used to screen by DHPLC the non-coding regions of the *MECP2* gene. Fragments 1, 13 and 14 are located in the 5′ UTR region, fragments 2, 3, 4 and 5 in intron 1, fragments 10, 11 and 12 in intron 2 and fragments 6, 7, 8 and 9 in the 3′ UTR region

PCR fragments	*(5′→3′) Oligonucleotides*	*Annealing temperature* (°C)	*Length* (bp)
Fragment 1	F1: CAAGTAGCTGGGATTACAGG R1: TTTGGGAGGCTGAAGCGGGT	62	160
Fragment 2	F2: TGGTAGCTGGGATGTTAGGG R2: TGGCACAGTTTGGCACAGTT	55	192
Fragment 3	F3: ATGGGTGACAGAGCAAGACT R3: ACTCCTGCCTGGGCGAACAA	55	200
Fragment 4	F4: GAATAACTTGAACCCGGCAG R4: TCCTACAACCCACAGAGCAG	55	174
Fragment 5	F5: AGGATTTCAGTGCAGGTTGG R5: ACTGCTGGAAAGGGAGCCAA	57	160
Fragment 6	F6: GTGACTTAGTGGACAGGGGA R6: TTTGAAGTGGGAACATGAAG	55	130
Fragment 7	F7: CTTAGAGTTTCGTGGCTTCA R7: GGGCACTGATGGCACCGAAA	55	287
Fragment 8	F8: CGTTTTCGGTGCCATCAGTG R8: GTGCCACTTTCCTGTCCTGT	58	240
Fragment 9	F9: ACCAGCCCCAARCCAAAACT R9: ATGTCACCAATTCAAGCCAG	57	476
Fragment 10	F10: AGCAACTCCTATCTCTACAG R10: CTGCTAACCTTTTTGGGATC	60	325
Fragment 11	F11: GAGCTAGGGGTTCAGAGGGG R11: CCAGGCCTCTCCAAAGTTCA	60	306
Fragment 12	F12: GCCTGACTCTTTGGTTGCTG R12: GACAAACAGAAAGACACAAGG	55	227
Fragment 13	F13: ATTGCAACTTCATTCAGCTG R13: GCTGCACGACCTTTTTCCCA	50	398
Fragment 14	F14: CCTTTATTTTAGCACTGTGT R14: AAGGTGTATTCTGGGGAGTC	60	255

Table 2 Primers used for the development of allele-specific amplifications method for each identified *MECP2* polymorphism

PCR fragments	*5′→3′ Oligonucleotides*	*Annealing temperature* (°C)	*Length* (bp)
7W	7W: CATGGGGGAAAGGTTTGGG**T** 3EF: TGCCCCAAGGAGCCAGCTAA	60	524
7V	7V: CATGGGGGAAAGGTTTGGG**C** 3EF: TGCCCCAAGGAGCCAGCTAA	60	524
8W	8W: CTTCTCTAAAGAATCCAACTGCCT**C** 3CF: TGCCTTTTCAAACTTCGCCA	53	1299
8V	8V: CTTCTCTAAAGAATCCAACTGCCT**G** 3CF: TGCCTTTTCAAACTTCGCCA	53	1299
9W	9W: CCCTGTCCACTAAGTCACA**G** 3AF: TGTGTCTTTCTGTTTGTCCC	57	2026
9V	9V: CCCTGTCCACTAAGTCACA**C** 3AF: TGTGTCTTTCTGTTTGTCCC	55	2026
10W	10W: GCAGAGGAACTTGCAGAGC**C** 3CR: TGAGGAGGCGCTGCTGCTGC	57	1213
10V	10V: GCAGAGGAACTTGCAGAGC**T** 3CR: TGAGGAGGCGCTGCTGCTGC	57	1213
12W	12W: GCAGTGTGACTCTCGTTCA**A** 3DR: TGGCAACCGCGGGCTGAGTC	64	1075
12V	12V: GCAGTGTGACTCTCGTTCA**G** 3DR: TGGCAACCGCGGGCTGAGTC	64	1075

X487C, one 677insA and one 1163del26. For each identified polymorphism, we have developed an allele-specific amplification. The oligonucleotide pairs used for PCR included one oligonucleotide specifically designed to hybridise with the variant type allele and one oligonucleotide with the wild type sequence in the gene. Each PCR fragment has been sequenced using internal primers to detect the presence or absence of the known *MECP2* mutation. The segregation of the variant and the pathogenic mutation allows us to determine the parental origin (Figure 2; Table 3). In five

Table 3 Results of the allele-specific amplification and sequencing for each individual informative family with the *MECP2* mutation and the single nucleotide substitutions of the *MECP2* gene. For the polymorphisms, position 1 corresponds to the A of the AUG codon in the cDNA sequence. A family was considered as informative when coherent segregation of the alleles is observed, and the mother and the daughter are heterozygotes for the polymorphic variant

RTT families	*MECP2 mutations*	*Nucleotide substitutions*	*Parental origin*
F2	R168X	378+266C→T	Paternal
F3	R294X	378+648C→T	Paternal
		1461+489G→C	
		1461+878C→G	
F7	X487C	1461+328G→A	Maternal
F8	R270X	378+266C→T	Paternal
F9	R294X	378+648C→T	Paternal
		1461+878C→G	
F10	1163del26	1461+489G→C	Paternal
F11	677insA	378+648C→T	Maternal
		1461+878C→G	

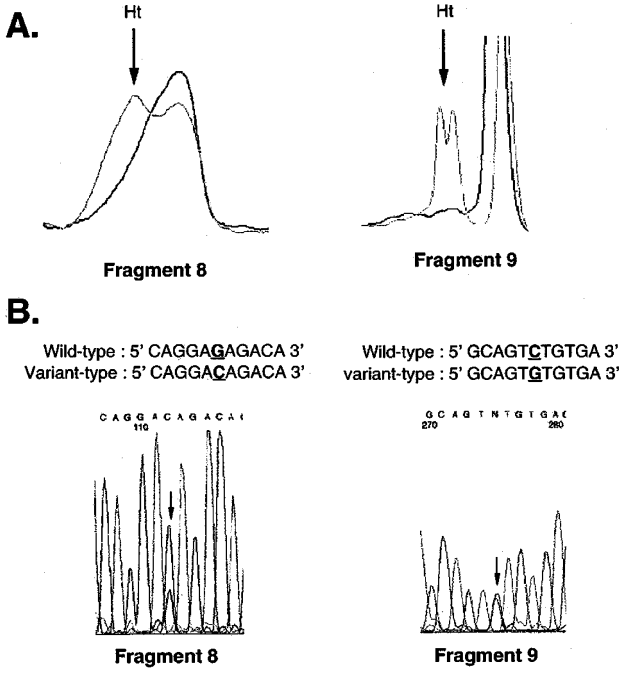

Figure 1 DHPLC detection of *MECP2* nucleotide variations. (**A**) Portions of elution profiles are shown for homoduplex and heteroduplex (Ht) peaks resulting from the analysis of PCR fragments 8 and 9. (**B**) Sequence analysis of PCR fragments 8 and 9 of the *MECP2* gene. The underlined nucleotides indicate the position of the polymorphic variants (1461+489G→C in fragment 8 and 1461+878C→G in fragment 9).

cases, the *de novo MECP2* mutations have a paternal origin (two R294X, one R270X, one R168X and one 1163del26) and in the two other cases a maternal origin (one X487C, one 677insA) (Table 3). In all transitions at CpG (C to T or G to A, when the 5-methylcytosine deamination occurs on the antisense strand), a paternal origin of the *de novo MECP2* mutation has been observed. In the pedigree in which a transversion occurred at CpG of the native stop codon (X487C), mutation occurred in female germ cells. For the two frameshift mutations, parental origin has been determined in one case and maternal origin in the other.

Discussion

Thirteen disorders were associated with an excess of affected female to male patients (Bloch-Sulzberger syndrome, OFD1 syndrome, Goltz syndrome, Aicardi syndrome, Rett syndrome, ...).[7] In most of these disorders, the discrepancy in the numbers of affected males and females still continue to be attributed to gestational lethality in males, though in most cases this hypothesis was not confirmed. Study of several recessive X-linked genetic diseases suggested that the deficiency of affected males could be related to a high ratio of male to female mutations. By use of molecular markers, direct evidence for a sexual bias in the origin of mutations has been shown for ornithine transcarbamylase deficiency, haemophilia A, haemophilia B and the Lesch-Nyhan syndrome. For example, in a study of 43 haemophilia B families, it was found that, while the male:female ratio of all point mutations was 3.5 : 1, the ratio of transitions at CpG dinucleotides was 11 : 1.[13] In the case of RTT syndrome, a dominant X-linked disease affecting only females, we show in this study that *de novo MECP2* mutations may have either paternal or maternal origin. In 71% of the cases, the *de novo MECP2* mutation has a paternal origin. All the analysed transitions at CpG (two R294X, one R168X, one R270X), which are estimated to account for 70% of mutations in the *MECP2* gene, have a paternal origin. This is compatible with previous data suggesting that methylation at CpG dinucleotides is reduced or absent in the female germ line.[14] Recently, results reported by Amir and colleagues using a more time consuming approach based on the analysis of somatic cell hybrids retaining either the maternal or the paternal X chromosome showed a paternal origin in two cases and a maternal origin in one sporadic case.[5] Moreover, data showing similar results were presented at the American Congress of Human Genetics in Philadelphia. From 26 sporadic cases with a clinical diagnosis of Rett syndrome, 23 have been shown to be from a paternal origin.[15] All these convergent data show a predominance of paternal origin mutations providing therefore a molecular explanation for the occurrence of the disease in most sporadic females. However, the occurrence of the mutation in maternal germ cells in the two cases suggests additional mechanisms for the sex-limited expression of Rett syndrome. It was proposed that the abnormal sex ratio of RTT was the result of early deaths of male foetuses.[16,17] As Rett syndrome is an

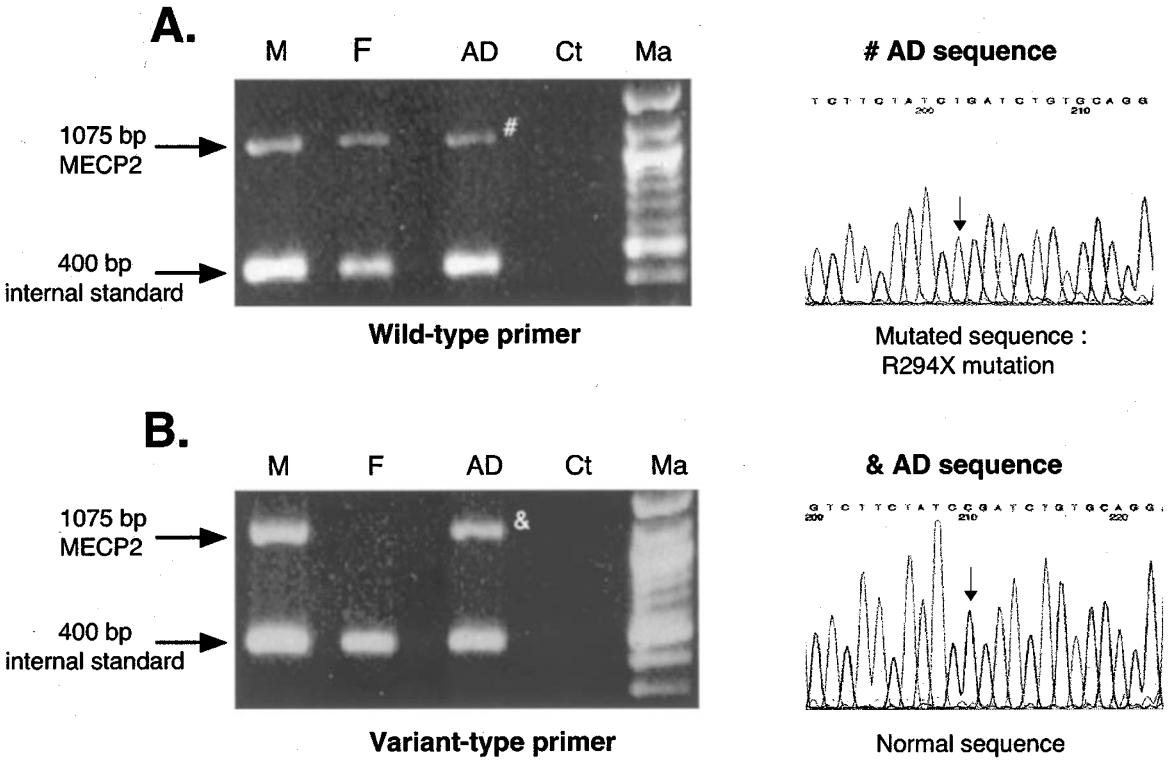

Figure 2 Example of a mutation of paternal origin. Agarose gel electrophoresis of amplified products corresponding to allele-specific PCR amplification and fluorescence sequence analysis of allele-specific PCR fragments of the *MECP2* gene. Each sample was separately amplified with the wild type (W) primer (**A**) and the variant type (V) primer (**B**). Coamplification of an internal standard fragment was also performed in each PCR reaction. The common primer used either with the normal or variant type allele-specific primer and the allele-specific primers for the 378+648C > T variant are indicated in Table 2 (3DR, 12W and 12V). M, unaffected mother; F, unaffected father; AD, affected daughter; Ct, negative control. Ma=100 bp marker (New England Biolabs).

X-linked dominant disease with (almost) every case arising as a new mutation, no such effect would be expected. Moreover, several studies have shown no increase in parental age,[18,19] or in spontaneous abortions rate among sibs.[20,21] It is not surprising, because such skewed sex ratio might be expected only when the mother is a healthy carrier of the *MECP2* mutation (perhaps spared by a skewed, favourable pattern of X inactivation) or bears the mutation in a mosaic state. Moreover, the sex limited expression of Rett syndrome is not complete. In fact, several recent reports described affected males presenting a *MECP2* mutation, but these affected males do not present a Rett syndrome phenotype.[22,23]

Screening for polymorphisms in the *MECP2* gene in combination with the development of allele-specific amplification of fragments encompassing the position of pathogenic mutations allowed us to reliably determine the parental origin of the *de novo* mutations associated with Rett syndrome in seven informative families. Although additional studies are required to reach statistically significant figures, our data suggest a predominant occurrence of *de novo* mutations in paternal germ line cells, providing therefore a relevant explanation for the predominant occurrence of Rett syndrome in females.

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