Identification of MeCP2 Mutations in a Series of Females with Autistic Disorder

Regina M. Carney, BS*, Chantelle M. Wolpert, GC, MBA*, Sarah A. Ravan, CGC†, Mona Shahbazian, BS‡, Allison Ashley-Koch, PhD*, Michael L. Cuccaro, PhD*, Jeffery M. Vance, PhD, MD*, and Margaret A. Pericak-Vance, PhD*

Rett disorder and autistic disorder are both pervasive developmental disorders. Recent studies indicate that at least 80% of Rett Disorder cases are caused by mutations in the methyl-CpG-binding protein 2 (MeCP2) gene. Since there is some phenotypic overlap between autistic disorder and Rett disorder, we analyzed 69 females clinically diagnosed with autistic disorder for the presence of mutations in the MeCP2 gene. Two autistic disorder females were found to have de novo mutations in the MeCP2 gene. These data provide additional evidence of variable expression in the Rett disorder phenotype and suggest MeCP2 testing may be warranted for females presenting with autistic disorder. © 2003 by Elsevier Inc. All rights reserved.

Introduction

In its classic form, Rett disorder (RTT, MIM312750) is an X-linked disorder characterized by normal neurologic development until 6 to 18 months of age, followed by a progressive developmental regression. RTT occurs almost exclusively in females. Incidence rates range from 0.25 to 1 in 10,000 female births [1,2]. Skjeldal et al. [3] reported a rate of 2.17 in 10,000 female births when the full spectrum of RTT syndrome variants was included. Developmental regression is followed by a deceleration of head growth, loss of purposeful hand movements, and followed by the appearance of midline, stereotypic hand movements [4]. Additional features observed in RTT patients include seizures, ataxia, hyperventilation, and social communication problems. These social engagement problems are similar to those observed in autistic disorder (AutD) and therefore can result in an initial misdiagnosis of AutD in some RTT patients [5]. Usually the appearance of the additional findings seen in RTT (Table 1) clarifies the diagnosis in these patients. Atypical RTT involves variations from the characteristic RTT profile in age of onset, sequence, and severity or presence of expected features. Consistently identified atypical groupings include congenital, forme fruste, and preserved speech variant, as well as other rare variants [6].

In 1999, Amir et al. [7] demonstrated that mutations in the X chromosome gene methyl-CpG-binding protein 2 (MeCP2) cause the majority of cases of RTT. The MeCP2 protein binds methylated cytosine residues, eventually leading to transcriptional repression [8]. Subsequent studies have found over 60 unique mutations in MeCP2, with mutations noted in 80-100% of RTT patients [9-12]. As previously mentioned, until recently RTT had only been observed in females and was believed to be lethal in males. However, since the identification of MeCP2 as the gene defect, two males with RTT have been reported [13]. In addition, increasing evidence points to a wider range of severity and heterogeneity of phenotype [14-17]. Further, Hammer et al. [18] indicate that MECP2 mutations result in a much broader clinical presentation than those encompassed under the RTT spectrum. In contrast to RTT, AutD occurs more frequently in males, with a male:female ratio of 4:1 [19]. However, studies have failed to demonstrate X chromosome linkage as a major genetic etiology for idiopathic AutD [20]. A study of the MECP2 gene in a...
small mixed gender group of children with AutD failed to
detect any mutations or polymorphisms [21]. This failure
led the investigators to conclude that MECP2 coding
sequences are not an important factor in autism. However,
the small number of females in the study limits the
findings. The purpose of the present study was to screen a
larger number of females diagnosed with AutD for muta-
tions in the MECP2 gene.

Materials and Methods

Family studies and patient evaluation

Two hundred and eighty-nine AutD patients from sporadic (one)
individual affected in a family and no family history of AutD) and
multiplex (two or more individuals with AutD in a family) families were
ascertained to identify AutD genetic risk factors. Sixty-nine of these
patients were female. Individuals included in this study were between 3
and 21 years of age and had a pre-existing clinical diagnosis of AutD.
Medical records were reviewed to confirm the medical and developmen-
tal history for each research participant. Individuals were excluded from
the study if they had one of the medical disorders known to be associated
with AutD (e.g., tuberous sclerosis complex, fragile X syndrome,
Smith-Lemli-Opitz syndrome, or structural brain abnormality) [22-25].

Also, AutD individuals with potentially confounding factors, such as
significant prenatal or perinatal events, were excluded as well. Likewise,
research participants were excluded if they had a history of meningitis,
head trauma, or lead poisoning. These factors, although not causally
associated with AutD, may significantly alter neurodevelopment in early
childhood, thereby complicating the clinical research evaluation and
possibly resulting in a phenotype similar to AutD [26]. Patients were
measured for head circumference; patients with microcephaly (< 2
standard deviations in head circumference) were excluded.

Research participants were also assessed for their level of adaptive
functioning using the Vineland Adaptive Behavior Scales (VABS) [27].
The VABS is a standardized measure of social, communication, and daily
living skills and consists of a semi structured interview with a primary
caregiver. Individuals with VABS-defined functioning of < 18 months
(age-equivalent) were excluded from any future analysis. Confirming
that an individual with AutD has a minimum level of adaptive function-
ing ensures the validity of the Autism Diagnostic Interview-Revised
(ADI-R) (age equivalent ≥ 18 months) [28]. The diagnosis of AutD was
confirmed by the ADI-R. The ADI-R follows a diagnostic algorithm for
AutD based on both DSM-IV and ICD-10 diagnostic criteria [4,28,29].

Informed consent was obtained from all participants, including care-
givers of all individuals with AutD included in this study. Ascertainment
of families and evaluation of affected individuals was conducted using
research protocols approved by the Institutional Review Board of the
participating institutions. All 69 AutD-diagnosed females were screened
for mutations in the MeCP2 gene.

Molecular analysis

Blood samples were collected, and genomic DNA was isolated from
peripheral leukocytes as previously described [30]. PCR primer pairs for
MeCP2 were based on those described by Bienvenu et al. [31]. DNA
samples were amplified in 50 μl final volume PCR reactions using 50 ng
the sum of the small and large (corrected) allele. The final XCI pattern factor (the ratio of allele densities from the undigested sample) was used to adjust the intensity of the large digested allele. The final intensity of the smaller allele divided by the intensity of the larger allele was calculated by dividing the intensity of the smaller allele to adjust the intensity of the large digested allele. Since the smaller allele amplifies more efficiently, a correction factor was included to increase the amplification specificity.

Initial screening of samples for MeCP2 mutations was performed by denaturing high performance liquid chromatography (DHPLC) (WAVE DNA-fragment analysis system, Transgenic, Omaha, NE)). WAVE-MAKER analysis software was used to estimate the optimal temperatures for the analysis based on known sequence. Several known MeCP2 mutations were used as positive controls for the analysis. Chromatographs that deviated from control samples were pursued for direct sequencing. Automated sequencing was performed on a CEQ 2000XL (Beckman-Coulter, Fullerton, CA). PCR products were purified using the QIAQuick PCR Purification Kit (Qiagen, Hilden, Germany). The intensity of the digestion. PCR products were separated by electrophoresis through a 6% denaturing polyacrylamide gel and exposed to a Phosphor screen (Molecular Dynamics, Amersham: Piscataway, NJ). The intensity of the bands was quantified using a Phosphorimager (Model Storm 860) with ImageQuant software (Molecular Dynamics, Amersham: Piscataway, NJ).

To analyze X chromosome inactivation (XCI) patterns, we used a modified protocol based on a previously described assay [32]. In summary, 1 μg of genomic DNA was digested with the methylation-sensitive restriction enzyme HpaII (Roche Molecular Biochemicals, Indianapolis, IN). 100 ng of either digested or undigested DNA was amplified by the polymerase chain reaction (PCR) using one 32P-labeled primer. The fragment amplified from the X chromosome contains an HpaII site as well as the highly polymorphic trinucleotide repeat of the androgen-receptor gene. Since the HpaII sites on the inactive X chromosome are methylated, this is the only allele that is amplified after digestion. PCR products were purposed using the QIAQuick PCR Purification Kit (Qiagen, Hilden, Germany). Reactions were run in both the forward and reverse directions (Dye Terminator Cycle Sequencing Kit, Beckman-Coulter, Fullerton, CA). The products of the sequencing reaction were cleaned using Edge Centriflex Gel Filtration Cartridges (Edge Biosystems, Gaithersburg, MD).

Whole genomic DNA, 1 X PCR buffer (Gibco, Carlsbad, CA), 0.2 mM dNTP, 1.5 mM Mg++, 5 U Taq Platinum polymerase (BRL, Carlsbad, CA), and 0.2 μg of each primer. Betaine (Sigma, St. Louis, MO; 1 M final concentration) was added to some reactions to increase the amplification specificity.

Results

Mutations in MeCP2 were found in two of the 69 AutD females studied. Neither patient meets all of the current diagnostic criteria for RTT (Table 1), based on the Rett Syndrome Diagnostic Criteria Work Group [33].

Patient 1

Developmental History. Patient 1 is a 16-year-old female. Her prenatal and perinatal course was unremarkable. She is the youngest of a sibship of three girls. There is no family history of mental retardation, RTT, or AutD. This patient developed normally until 18 months when her parents noted that her speech stopped developing, although language milestones for first words and phrased speech were achieved on time. Patient 1 was assigned a diagnosis of AutD at age 8 years, 9 months. There is no history of bruxism, no respiratory difficulty, and no history of toe-walking.

Clinical Evaluation. This female patient has a minimal response to verbal stimuli. However, she uses simple phrases to communicate her wants and needs. She does not have any neologisms, pronoun reversals, or verbal rituals. On physical examination, this patient’s head circumference is at the fiftieth percentile. Initial head circumference was within normal limits. When walking, the patient holds her left arm flexed at the elbow and wrist, compatible with dystonic posturing. She also occasionally exhibits side-to-side stereotypic hand movements. There are no hand-wringing, clapping, or other stereotypic hand movements. No truncal ataxia was observed, and her gait is normal-based. No scoliosis was present.

Administration of the VABS [27] indicated a current level of functioning equivalent to 60 months. The patient met DSM-IV diagnostic criteria for AutD as confirmed by the ADI-R.

MeCP2 Analysis. Sequencing of the patient’s MeCP2 gene demonstrated a heterozygous 41-bp deletion at nucleotides 1157-1197 (numbered from the start of the coding region; Fig 1). This 1157del41 mutation causes a frameshift resulting in a MeCP2 protein truncated to

![Figure 1](96x601 to 516x762)

Figure 1. Sequencing of Patient 1: PCR products were cloned to allow separate analysis of each chromosome. Line A demonstrates a 41-bp deletion, noted by the arrows, beginning at nucleotide 1157 and extending to bp 1198. The sequence of deleted nucleotides is highlighted in black. Line B shows the sequence of the other normal chromosome, which matches the GENEBANK (consensus) sequence shown in line C. Chromatographs are shown in line E (normal sequence) and line D (deletion). The arrows denote the same sequence point in both chromosomes, flanking the deleted area. The family members of Patient 1 demonstrated no change on sequencing, indicating that this is a de novo mutation.
389 amino acids from the normal 486. This mutation has previously been reported in two females with classic RTT [34,35]. The sequence of the MeCP2 gene in the patient’s two sisters, mother, and paternal grandparents was normal (data not shown). The father was unavailable for analysis.

**Patient 2**

*Developmental History.* Patient two is a 10-year-old female who had a normal prenatal and perinatal course. She presented at age 10 months with early morning waking and hyperactivity. This behavior continued, and concomitantly the patient acquired her first single words at age 12 months and started speaking in two-word phrases at age 24 months; motor milestones were achieved on time. At age 30 months the patient experienced a regression that included the loss of language, motor skills, and adaptive behavior. An intermittent, nonspecific electroencephalogram (EEG) abnormality was noted in the patient. Patient 2 was diagnosed with AutD at age 42 months.

*Clinical Evaluation.* Patient 2 responds appropriately to verbal stimuli with one-word answers. Her one-word responses are understandable to her caregivers but not to strangers. Patient 2 communicates with simple phrases to voice her wants and needs. She does have some use of neologisms and verbal rituals. Her head circumference is at the fortieth percentile. Her head circumference measurement at birth is not available. She does not have dysmorphic features. She has left esotropia. Patient 2 has diminished grip strength. She was able to grasp the examiner’s hands on command but the examiner’s hands could be easily withdrawn. Her gait was wide-based at this time, with progressive changes as she was reported to have been able to tandem gait at 5 years of age. On examination she did not exhibit the stereotypic hand movements normally associated with RTT, such as wringing, clapping, or clenching. There was no history of bruxism, oculegic crises, or scoliosis. Administration of the VABS [27] reveals that she functions at a level equivalent to 32 months of age. The patient met DSM-IV diagnostic criteria for AutD as confirmed by the ADI-R [28].

*MeCP2 Analysis.* Sequencing indicates that this patient has a single nucleotide sequence change 880 C→T (Fig 2). This nonsense mutation (R294X) is one of the most frequent mutations found in RTT [11,31,34-40]. The father, mother, and brother were not found to carry the change, confirming that this is a de novo mutation.

*X chromosome inactivation*

Since the mutations identified in the two patients are both commonly found in individuals with classic Rett syndrome, we tested whether the milder phenotype may be due to a skewing of X chromosome inactivation. Analysis of XCI in peripheral blood leukocytes from the two patients revealed borderline skewing. Patient 1 demonstrated a 31% pattern and Patient 2 exhibited a 29% pattern. The XCI ratio is considered skewed when it is 20% or less (or 80% or greater) [41].

**Discussion**

Mutations in the MeCP2 gene were identified in two females (ages 10 and 16) who meet criteria for the diagnosis of AutD. The diagnosis of AutD in these patients was confirmed using the ADI-R. This psychometric instrument is the current “gold standard” for diagnostic confirmation in all ongoing genetic linkage studies for AutD [29,42-45].

Neither of these patients evaluated exhibit the classic RTT features [4]. The possibility of a RTT variant [46,47] was examined given the findings of developmental changes and delays. Using the criteria for Rett variants as defined in the Hagberg and Skjeldal model, neither participant satisfied the full criteria for atypical Rett syndrome. However, there were clear manifestations of developmental abnormality that included perturbations in developmental sequence of language, adaptive behavior, and cognition. Kerr et al. [14] have recently described a scoring system for reporting on clinical features in individuals with MeCP2 mutations. Although no normative data are available, it is noteworthy that our two individuals have scores that may be interpreted as in the lower range of the RTT spectrum (Kerr = 6 and 7 respectively, for patients 1 and 2). This interpretation is based on the Kerr
The pattern of adequate early development followed by regression in language and social functioning is common in both AutD and RTT. The data presented here highlight the clinical overlap between RTT and AutD, particularly in early childhood. This overlap has been recognized for several years. Gillberg [5], on the subject of this overlap, suggested that the development of “microcephaly, typical loss of hand-function and ‘hand-washing’ stereotypies” usually serve to distinguish the two as the child develops. However, in a later report he demonstrates five cases initially diagnosed with AutD that later developed additional findings more consistent with RTT [48]. Interestingly, he states that “it is reasonable to assume that what lies behind this overlap of symptoms might be a common neurobiological substrate.” He adds that RTT might represent a condition with “considerable phenotypical (and perhaps etiological) heterogeneity.” Other investigators, including Andreas Rett [49], have also attempted to differentiate the two entities in early childhood by the absence or presence of several clinical factors.

The MeCP2 findings in the two girls with AutD demonstrate that the phenotypic heterogeneity of RTT is quite variable. The variation clearly extends beyond RTT, and MeCP2 mediated disorders should be suspected in the presence of nonspecific mental retardation. Recent investigators have reiterated this as well [15-17]. Indeed, our data reveal that a clear clinical divergence of RTT and AutD in later childhood does not necessarily occur. Despite overlapping phenotypes, the underlying genetic etiologies of RTT and AutD differ. Thus, genetic heterogeneity is a confounding factor both in patient care settings and ongoing genetic research studies.

Only two studies to date have investigated MeCP2 mutations in the AutD population. Lam et al. [35] screened 21 patients with “autism and mental retardation” and found one patient with an MeCP2 mutation: a 4-base pair deletion in the 5’; splice site of intron 2 (1p52 + 2 del TAAG). However, they were unable to confirm this change in mRNA. Vourc’h et al. [50] failed to identify mutations in the MeCP2 coding sequence in a sample of 59 patients with autism, only 17 of which were females. Both of the mutations described in the current study have been noted in classic RTT patients. Therefore, additional factors, such as X chromosome inactivation and modifier genes, must be involved in the clinical variability.

Molecular genetics influences the diagnostic boundaries of many disorders, where different or variable clinical presentations are due to mutations in the same gene. In some cases, such as Fragile X syndrome, molecular findings are central to the diagnosis. For other disorders, like Rett disorder, the diagnosis remains a clinical decision, although eventually genotype-phenotype correlation data may facilitate diagnosis and prognostication based on molecular genetic findings.

Do the results presented here suggest that all female AutD patients should be tested for MeCP2 mutations? Kerr et al. [14] suggest that individuals in late childhood with developmental delay and autistic tendencies may be candidates for MeCP2 testing but do not discuss AutD females specifically. Hammer et al. [18] encourage screening for MeCP2 mutations in nonspecific cases of MR and suggest that genetic counseling is warranted. The overlap of mental retardation in approximately 70% of individuals with autism supports exploration of MeCP2 in these individuals. Astute use of genetic testing for MeCP2 mutations by clinicians may lead to a reduction or elimination of further time-consuming and expensive tests (e.g., fluorescent in situ hybridization testing for Angelman syndrome). For the family, the importance of a definitive diagnosis cannot be underestimated. The greatest benefit of MeCP2 testing may be for genetic counseling. Even though most classical Rett cases are sporadic (99.5%), the percentage of atypical cases that are familial has not been clearly established. For example, Couvert [51] examined MeCP2 mutations in XLMR and found familial mutations in two of six families with identified mutations. Considering that many atypical Rett cases have likely not been ascertained/identified because of deviations from the classical phenotype, the true percentage that are familial remains to be established. The clinical benefits of MeCP2 testing include provision for assessment of recurrence risk, identification of repro-
ductive options (i.e., prenatal diagnosis), and molecular assessment of siblings that may lead to earlier diagnosis and treatment. The latter are generally thought to enhance outcomes. Finally, it is not clear how individuals with AutD differ clinically from individuals with AutD and the MeCP2 mutation. The utility of this distinction may lie in research that demonstrates individuals with Rett syndrome and AutD respond differently to treatment [52]. Thus genotyping results could have implications on the appropriate therapeutic usage of medical and psychosocial management as well.

**Conclusion**

These data suggest that the phenotype associated with MeCP2 mutations is highly variable, and that individuals with MeCP2 mutations can present with a milder clinical phenotype such as AutD. Thus, in some cases, MeCP2 testing may be warranted for females presenting with AutD, especially those presenting with any associated symptoms of RTT.

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210 PEDIATRIC NEUROLOGY Vol. 28 No. 3


