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Detection of Rarely Identified Multiple Mutations in *MECP2* Gene Do Not Contribute to Enhanced Severity in Rett Syndrome

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Abstract

The objective of our study was to characterize the influence of multiple mutations in the *MECP2* gene in a cohort of individuals with Rett syndrome. Further analysis demonstrated that nearly all resulted from *de novo in cis* mutations, where the disease severity was indistinguishable from single mutations. Our methods involved enrolling participants in the RTT Natural History Study (NHS). After providing informed consent through their parents or principal caretakers, additional molecular assessments were performed in the participants and their parents to assess the presence and location of more than one mutation in each. Clinical severity was assessed at each visit in those participants in the NHS.

Non-contiguous *MECP2* gene variations were detected in 12 participants and contiguous mutations involving a deletion and insertion in three participants. Thirteen of 15 participants had mutations that were *in cis*; four (of 13) had three *MECP2* mutations; two (of 15) had mutations that were both *in cis* and *in trans* (i.e. on different alleles). Clinical severity did not appear different from NHS participants with a single similar mutation.

Mutations *in cis* were identified in most participants; two individuals had mutations both *in cis* and *in trans*. The presence of multiple mutations was not associated with greater severity.

Nevertheless, multiple mutations will require greater thought in the future, if genetic assignment to drug treatment protocols is considered.

Keywords

Rett Syndrome; *MECP2*; *in cis* Mutations; clinical severity

INTRODUCTION

Multiple mutations within the same allele of a gene although rare, have been identified in many human diseases [Chen et al., 2009]. These disease-causing mutations can stem from numerous genetic alterations; including single nucleotide substitutions and deletions/insertions of single nucleotides or large stretches of DNA. Whether multiple mutations reside on the same chromosome (*in cis*) or different chromosomes (*in trans*), is of importance in understanding the impact on gene function and possibly the clinical phenotype [Kelly and Semsarian, 2009]. *In cis* mutations have been further categorized as noncontiguous or contiguous: noncontiguous is defined as two or more mutations existing with at least two non-adjointing nucleotide changes, while contiguous describes two or more mutations that are comprised of adjoining nucleotide changes. Empiric evidence indicates that gene history at a given locus is the major factor, rather than variation in the rate of local mutations suggesting that *in cis* mutations occur more frequently than *in trans* mutations [Reich et al., 2002].

Examples of disorders that have been associated with multiple mutations, both familial and de novo, include: genetic variations of cardiovascular disease [Blair et al., 2001; Kelly and Semsarian, 2009], familial hypercholesterolemia [Kotze et al., 1997], cystic fibrosis [Hicks et al., 2003], hypochondroplasia [Pannier et al., 2009; Santos et al., 2007], biotinidase deficiency [Norrsgard et al., 1998], Marfan syndrome (*de novo*) [Wang et al., 1996], androgen insensitivity syndrome (*de novo*) [Mongan et al., 2002], and a variety of inherited diseases occurring within isolated populations [Zlotogora, 2007] (for further disorders see Chen et al. [2009]). Due to their rarity, the progressions of diseases associated with multiple mutations of the same gene are not well understood. It is hypothesized that these unique genetic conditions lead to disease states of variable phenotypic outcomes [Botstein and Risch, 2003].

One disease where multiple *de novo* mutations in a single gene have been identified is Rett syndrome (RTT; OMIM #312750; <http://www.ncbi.nlm.nih.gov/omim/>) [Bunyan and Robinson, 2008; Weaving et al., 2003]. RTT is the leading cause of severe cognitive impairment in females, with approximately 1:10,000 females worldwide affected by this disorder. RTT is characterized by apparently normal early development until 6-18 months of age when developmental delay occurs with stagnation of growth and motor, language, and social skills. A period of rapid developmental regression follows, which may extend up to age 4 years [Percy, 2002]. Thereafter, improvement in eye contact and social skills occur that remain stable throughout life. During periods of developmental regression, features of autism including limited eye contact and poor socialization occur [Percy, 2011]. Other features of the disorder include stereotypic hand movements, periodic breathing episodes, scoliosis, gait dysfunction, dystonia, and increasing rigidity in later years of life [Percy and Lane, 2005].

RTT was first described by Dr. Andreas Rett in 1965 and is currently diagnosed by clinical criteria based on core features [Neul et al., 2010]; [Percy et al., 2010]. In 1999, mutations in the *MECP2* gene encoding methyl-CpG-binding protein 2 (OMIM 300005), located on the X chromosome at Xq28, were linked to the disorder and are now detected in more than 95% of individuals with RTT [Amir et al., 1999]. MeCP2 is characterized as a transcriptional modulator, demonstrating both transcription repressing and activating functions [Chahrour et al., 2008]. While mutations in *MECP2* are highly correlated with the diagnosis of RTT, the specific functions of MeCP2 that are impaired and are responsible for RTT symptomatology remain unknown. In the majority of families, RTT is due to *de novo* germline *MECP2* mutations in the paternal X chromosome [Trappe et al., 2001]. However, in a small number of families, patterns of inheritance have been discovered where *MECP2*

mutations are present throughout multiple generations [Augenstein et al., 2009]. To date, more than 250 mutations have been identified in *MECP2*, but eight specific mutations account for more than 60% of individuals with RTT [Percy et al., 2007]. Interestingly, mutations of *MECP2* have been implicated in other neurological disorders, including schizophrenia [Cohen et al., 2002].

Studies describing multiple *MECP2* mutations have only represented 12 patients thus far, indicating either the rare occurrence of this phenomenon, or an underreporting of multiple mutations in this disorder [Bunyan and Robinson, 2008; Weaving et al., 2003; Fendri-Kriaa et al., 2011; Fendri-Kriaa et al., 2012]. These reports described *in cis* mutations of the *MECP2* gene in one instance and were assumed, although not confirmed, to be *in cis* in the others [Bunyan and Robinson, 2008]. Here we characterize the largest cohort so far referred for *MECP2* genetic analysis demonstrating multiple mutations on the *MECP2* gene. Further analysis demonstrated that 8 resulted from *de novo in cis* mutations. The disease severity was indistinguishable from that of single mutations. While the frequency of multiple mutations appears very low, further study could provide insight illuminating *MECP2* gene hotspot mutations at CpG sites, future treatment options, and genotype-phenotype relationships.

METHODS

Individuals participating in this study were enrolled in the Rare Diseases Clinical Research Network (RDCRN), of which one consortium is dedicated to studying the natural history of Angelman, Prader-Willi, and Rett Syndromes (<http://rarediseasesnetwork.epi.usf.edu/arpsc/studies/Rett-5201.htm>). Participants were enrolled in the RTT Natural History Study (NHS) after providing informed consent through their parents or principal caretakers. The NHS is a multi-center based study, so for that reason, institutional review board approval was obtained at each institution-based site. The NHS is registered as clinical trial NCT00296764.

These individuals have been seen semi-annually to age 6 and annually thereafter [Percy et al., 2010]. In addition, two quantitative measures of clinical status were completed by the study physicians to assess participants' severity; the Clinical Severity Scale (CSS) and the motor behavioral analysis (MBA) [Lane et al., 2011]. The CSS is based on diagnostic and developmental features for RTT, which include the age at onset of regression, hand stereotypies, and the degree of deceleration of head growth. The MBA is a broader spectrum analysis, which examines motor, behavior, and respiratory dysfunction. To determine if function was affected by the type of mutations, data from these quantitative behavioral measures were grouped together by mutation type and were analyzed statistically using the unpaired Student's t-test (Microsoft®, Excel®, Redmond, WA). $P < 0.05$ was considered significant.

Although genetic testing is required prior to entry into the RTT NHS, after obtaining informed consent, DNA was isolated from both the individuals diagnosed with RTT and their parents in order to confirm the presence of the multiple mutations initially reported. Genomic DNA was isolated from peripheral blood leukocytes by standard protocols (ArchivePure DNA Isolation Kit; 5 Prime, Gaithersburg, MD). As the mutation status was previously determined, polymerase chain reaction (PCR) analysis of the *MECP2* exon / exon-intron boundary of interest was completed using primers designed in the Molecular Diagnostics Laboratory. The PCR products were purified (Qiagen Inc., Valencia, CA), sequenced using the Big-Dye Terminator v3.1 kit (Applied Biosystems, Foster City, CA), and electrophoresed on an ABI 3130XL Genetic Analyzer (Applied Biosystems). Nucleotide sequences were compared to the control reference sequence (NM 004992.3) using

MacVector and Assembler software (MacVector Inc., Cary, NC). Once the presence of two or more variants was confirmed, a method was developed to separate the alleles: either PCR and enzyme digest or PCR and subsequent cloning.

In participants where restriction sites were created or abolished (Table I, Participants 3 and 7), PCR products were digested with specific enzymes and separated by gel electrophoresis. PCR bands were excised and purified by gel extraction (Qiaquick Gel Extraction Kit, Qiagen) prior to sequencing. The restriction enzyme cut the PCR fragment at one of the *MECP2* mutation sites, allowing separation of the mutant and normal alleles at this locus. By isolating and sequencing the mutant and normal PCR products, we determined whether the second mutation was located *in cis* or *in trans* of the first mutation.

In cases where a restriction enzyme could not be utilized, subcloning of PCR amplified regions was performed. Briefly, PCR products were cloned into the pCR4-TOPO plasmid vector using the One Shot TOPO TA Cloning Kit for Sequencing (Invitrogen Inc). The PCR products were inserted into bacterial plasmids, and the bacteria were grown on LB (Luria-Bertani broth) agar plates with carbomycin at a 50ng/ml concentration for 24 hours. Colonies were picked and screened for correct insert size via PCR with M13 primers. Only those colonies with the correct insert size were expanded in LB broth with carbomycin at 60ng/ml for 14-16 hours. After plasmid preparations, samples were purified with the QiaQuick MiniPrep Kit (Qiagen) and sequenced with M13 primers. Bi-directional-sequencing was performed with Big Dye Version 3.1 (Applied Biosystems). Chromatograms were visually inspected before alignment to the *MECP2* reference sequence using MacVector and Assembler software. Multiple clones were sequenced to detect the normal and mutant alleles from patients with more than one mutation.

RESULTS

Table II provides a detailed description of the non-contiguous *MECP2* gene variations detected in the twelve participants and, where possible, their parents. Table III presents similar information on the contiguous mutations involving a deletion and insertion in three participants. In terms of contiguous mutations that we identified (Table III), all represent a deletion and insertion occurring at the same site. Contiguous mutations can represent many alterations, and in each instance we classify these mutations as contiguous because in all cases the number of nucleotides deleted does not equal the number of nucleotides that are inserted, presumably representing two different processes. We present these not to define their functional status, but to provide observation to a unique genetic modification. Figure 1 is a schematic representation of *MECP2* gene mutations identified in our cohort. The designation of these mutations is derived from published nomenclature [Antonarakis, 1998; den Dunnen and Antonarakis, 2000].

The mutations detected in many of the patients in our cohort have been described elsewhere. Mutations were compared to entries in the comprehensive database maintained by the International Rett Syndrome Foundation (IRSF) and the Children's Hospital of Westmead (RettBASE: IRSF *MECP2* Variation Database, <http://mecp2.chw.edu.au/cgi-bin/mecp2/search/nd-search.cgi?form=single>). We did identify the following novel nucleotide changes (insertions/deletions) not previously described (Listed in Tables II and III): c. 488_995del1508, c.855_859del4, c.855ins12, c.1024ins2, c.1026_1029del4, c. 1061_1062delGC, c.1089_1129del41, c.1094_1138del45, c.1161_1166del6, c. 1180_1205del26, and c.1156_1197del42. Furthermore, the *MECP2* polymorphism located in intron 3, c.378-109A>G, was recently added to the SNP database-rs3850326.

Of the more than 800 females with RTT in the NHS, we confirmed only 15 having more than one *MECP2* mutation (less than 2% of our population). All but two had mutations that were *in cis* (Tables II and III). Three females had three *MECP2* variations (Table II; Participants 1, 9, and 11); two individuals (Participants 1 and 2) had mutations that were *in trans*, i.e. on different alleles. Most of the mutations identified were located in exon 4. The only exception was participant 6 whose mutations were located in exon 3 and 4, namely a premature stop codon (p.Ser68*) in exon 3 and a deletion of 15 amino acids downstream in exon 4.

Parents' DNA was sequenced and available for analysis in 10/15 participants; both parents in eight participants and just mothers in two participants (participants 8 and 9). Of the group, two mothers and two fathers were positive for a *MECP2* mutation; the remaining parents were negative. We also had DNA sequence information for four other participants with the report of two mutations. However, for these individuals, the mode of inheritance patterns or the nature of the alleles that were mutated was not determined, and the reported mutations were not confirmed as we only had their reports and were not able to obtain parental information.

In three instances, parental inheritance was a contributing factor (Fig. 2). Participant 12 carries *in cis* a *de novo* mutation at amino acid position 134 resulting in mutation p.Ser134Cys and an in-frame polymorphism (c.1168_1173del CCACCT, rs61753008) deleting prolines at positions 390 and 391. The mother was negative for the mutation and polymorphism, while the father carried the rs61753008 polymorphism (Fig 2B). Parental inheritance was identified for two additional participants' mutations and polymorphisms (Fig 2C and 2D). Participant 1 had a nonsense mutation (p. Arg168*) and a c.1180G>A polymorphism (resulting in amino acid change p.Glu394Lys) on one allele, and a second mutation, p. Ala278Thr, on the other allele (Fig 2C). The father also exhibited this polymorphism, indicating a *de novo* mutation for the p. Arg168* change. The mother carries the p.Ala278Thr mutation, and upon further investigation, an X-chromosome inactivation (XCI) pattern that was random (64:36). Participant 2 had a deleterious mutation (p.Gln227Leu) on one allele (Fig 2D). A polymorphism, c.378-109A>G and an additional mutation (p. Pro173Arg) are on the other allele. The mother was identified as a carrier of the p. Pro173Arg mutation and her XCI pattern was also random (79:21), where a distribution ratio of >80 :< 20 reflected as non-random pattern. While the 79:21 ratio lies very closely to the important ratios of >80 :< 20, it still suggests random patterning, as determination of these percentages as 20% or less or 80% or higher has been previously described [Amir et al., 2000]. Unfortunately, for Participant 2, XCI was uninformative.

To determine if multiple mutations on the *MECP2* gene affect clinical severity for these 15 participants, in the NHS we used two types of assessments of clinical severity, the CSS and MBA, which we obtained from every participant at each visit to our clinic (4-10 on each scale). We compared the participants' score on these two items with corresponding items from our database related to the proximal mutation in each instance (Table IV). We could not identify any significant difference for those individuals with more than one mutation compared to those individuals with a single mutation.

To examine whether multiple mutations of the *MECP2* gene affect functional differences and clinical severity, data from individuals were grouped according to the types of mutation that were present. Mutations that result from a rearrangement of the gene which occurs with large frameshifts or nonsense mutations (Large-scale protein alterations: Participants 4, 6, 7, 8, 9, 10, 11, and 12) were grouped together and compared to individuals with two missense mutations or a missense mutation and a deletion of one amino acid (Single amino acid alterations: Participants 1, 2, 3, and 5). The CSS for the large-scale protein alteration group

was 21.6 (mean), 6.6 standard deviation (S.D.), where $n = 8$ versus the single amino acid alteration group which was 21.9 (mean), 6.9 S.D., where $n = 4$; $P = 0.94$. The MBA for the large-scale protein alteration group [43.6 (mean), 12.0 S.D., where $n = 8$ versus the single amino acid alteration group 43.4 (mean), 12.8 S.D., where $n = 4$; $P = 0.97$] did not differ in individuals with large frameshift or nonsense mutations when compared to individuals with two missense mutations or a missense mutation and a deletion of one amino acid. These data suggest that the type of *MECP2* mutation within multiple mutations do not contribute to worsening outcomes and a more severe phenotype is presumable dictated by other factors including XCI and the specific location and nature of individual mutations.

DISCUSSION

While multiple mutations in single disease-producing genes are rare, understanding their frequency could provide insight into susceptibility in terms of vulnerable genes and nucleotide regions. We re-analyzed 12 participants, including 18 parents, followed through the RTT NHS characterizing multiple mutations in the *MECP2* gene. This group represents the largest study on this subject to date [Percy et al., 2010]. An analysis of multiple mutations on the same or different alleles should provide information regarding the association with altered disease progression and could reveal more about genotype/phenotype relationships.

Mutations in *MECP2* have been identified in >95% of individuals with RTT and are predominantly caused by C > T transitions at 8 CpG dinucleotides located in the third and fourth exons of the *MECP2* gene [Percy, 2011]. However, the specific function of MeCP2 impaired by mutations and responsible for RTT symptomatology is undetermined. We described a number of novel mutations that had not been reported previously, adding to the 250 mutations already reported in *MECP2* (<http://mecp2.chw.edu.au/mecp2/>). Multiple mutations in the *MECP2* gene account for roughly 2% of the RTT population that we used in this study. While this represents a very small percentage of the total population, it does provide clear evidence to screen the complete *MECP2* gene for multiple causative mutations.

As described elsewhere, the occurrence of more than one mutation in a single gene is a rare event [Kondrashov, 2003]. The significance of multiple mutations on disease impact remains unknown, particularly in terms of a worsening clinical phenotype [Chen et al., 2009]. In this report, we were unable to identify any significant difference for those individuals with more than one mutation compared to those individuals with a single mutation in two different indices of clinical severity, the MBA and the CSS. It had been speculated that mutations occurring *in trans* might produce a more deleterious clinical phenotype than those involving a single allele [Gratzer, 1994].

Only two case studies and two case reports have been published describing multiple mutations in the *MECP2* gene [Bunyan and Robinson, 2008; Weaving et al., 2003; Fendri-Kriaa et al., 2011; Fendri-Kriaa et al., 2012]. The authors in the two case studies speculated that the phenotype associated with more than one mutation in the gene showed a trend toward a worse phenotype [Bunyan and Robinson, 2008; Weaving et al., 2003]. Weaving et al. speculated that having more than one mutation can impact disease outcome without regard to the position of the mutations. While Bunyan and Robinson [2008] found that *in cis* mutations were confirmed in one individual while three additional individuals were presumed to be *in cis*. These were not regarded as producing a significantly different phenotype.

In the present study, we found *in cis* mutations in most participants, but we did identify two in which mutations occurred both *in cis* and *in trans*. Understanding *in trans* mutations can have a great impact in determining how the genotype could alter disease progression. It is presumed that *in trans* mutations would have a major impact on disease severity, as no mechanisms would be able to compensate for the defect in one allele, such as XCI. If mutations in both alleles occur that are of significant impact to the function of the protein, embryonic lethality or a more severe phenotype would occur that would be quite different from other individuals with RTT and would not necessarily be tested for *MECP2* mutation analysis. In both instances, however, only a single mutation was found to be significant. Each mother and one father carried the other mutations. These were regarded as polymorphisms as the parents were all considered to be normal and each mother had random XCI. It could be hypothesized that these results could be a consequence of how the XCI studies are conducted, as peripheral blood is used to determine the genetic status of cells in the central nervous system. However, results have shown that RTT patients have balanced XCI patterns in peripheral blood compared to XCI patterns in the brain, and for that reason can be used as an accurate indicator [Shahbazian et al., 2002]. Thus some mechanism exists that causes a single mutation to be significant and future research in various laboratory and clinical settings needs to address how particular mutations and DNA rearrangements in the *MECP2* gene affect phenotypic outcomes.

Determining multiple mutations within a single gene provides a novel finding and a genetic oddity; however categorizing these mutations might play a bigger role in the future when pharmacological therapy might rely on genetics. For example, aminoglycoside-like compounds have a unique pharmacological feature that allows them to read-through premature stop sequences, and has shown promise in models of RTT [Brendel et al., 2011]. However, a patient with two pathogenic mutations in the same gene, one a missense and one a nonsense mutation, might not completely respond to read-through therapy as this treatment is only altering half of the mutations. Thus extreme care and greater thought needs to be applied to multiple mutations in the future, especially if genetic utilization of drug regimens can be established. Furthermore, examining the location and specific nature of multiple mutations within a single gene could provide a greater understanding of gene instability. Lastly parental screening of probands with multiple mutations could be used to determine differences in the molecular composition of the *MECP2* gene that could be used by researchers to predict sites of the gene that have a high probability of mutation.

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Figure 1. ARRAYED ILLUSTRATION OF *MECP2* INDICATING THE REGIONS INCLUDING THE FOUR EXONS AND VARIOUS DOMAINS

MECP2 is arrayed indicating the regions including the four exons and the C-terminal domain. The positions of mutations identified in this study are noted: missense mutations above the diagram and nonsense mutations are indicated below the diagram.

Abbreviations: NTD = N-terminal domain; MBD = Methyl-binding domain; ID = Inter-domain; TRD = Transcription-regulating domain; CTD = C-terminal domain; ATG = Start codon; TGA = Stop codon

Figure 2. PEDIGREE EXAMPLES OF INDIVIDUALS WITH NONCONTIGUOUS MECP2 SEQUENCE ALTERATIONS

Four examples of individuals with noncontiguous *MECP2* sequence alterations. a. A participant with a *de novo* mutation and an allele with no change; b. A participant with a paternally inherited *de novo* mutation and a single nucleotide polymorphism (SNPrs61753008); c. A participant with a paternally inherited *de novo* mutation (p.Arg168*) and a maternally inherited p.Ala278Thr mutation and a SNPrs63094662; and d. A participant with a paternally inherited *de novo* p.Gln227Lys mutation and a p.Pro173Arg mutation and a SNP rs3850326 inherited from the mother.

TABLE I
RESTRICTION ENZYMES IN SPECIFIC *MECP2* MUTATIONS

Table I shows examples of how the restriction enzymes cut at the site of one mutation, separating the two alleles at this locus. This method allowed for sequencing determination of *in cis* or *in trans* of the two mutations.

	Enzyme	Enzyme Site	Digest results
Participant #3	BclI	CCATC(N) ₄	Cuts at c.905C but not the c.905T position
Participant #7	SacI	GAGCTC	Cuts normal allele within c.1157_1197 region

TABLE II
MECP2 MUTATIONS: NONCONTIGUOUS

Individuals with more than one noncontiguous nucleotide change in the *MECP2* coding sequence were identified and characterized. A number of these had large mutations involving single complex deletions or deletions/insertions.

Participant	Mutations cDNA level	Mutations Amino Acid level	Maternal Information	Paternal Information
1 *	c.502 C>T c.832 G>A c.1180G>A	p.Arg168 * p.Ala278Thr p.Glu394Lys (SNPrs63094662)	Positive for c.832 G>A; p.Ala278Thr	Positive for c.1180G>A (p.Glu394Lys) (SNPrs63094662)
2 **	c.378-109G>A c.518 C>G c.675_678delT	c.378-109G>A (SNP rs3850326) p.Pro173Arg p.Gln227Lys	Positive for c.518 C>G; p.Pro173Arg and c.378-109G>A (SNP)	Negative for mutations
3	c.905 C>T c.917 G>A	p.Pro302Leu p.Arg306His	Negative for mutations	Negative for mutations
4	c.1161_166del6 c.1180_1205del126	p.Pro390_391del p.Glu394Pro_fsX1	Negative for mutations	Negative for mutations
5	c.502 C>T c.880 C>T	p.Arg168 * p.Arg294 *	Negative for mutations	Negative for mutations
6	c.203 C>G c.1094_1138del145	p.Ser68 * p.Glu365_Pro379del	Negative for mutations	Negative for mutations
7	c.1104_1106del3 c.1157_1197del41	p.His372del p.Leu386Hisfs * 4	Negative for mutations	Negative for mutations
8	c.1089_1129del41 c.1156_1197del42	p.Lys364Glyfs * 12	Negative for mutations	Not done
9	c.1024ins2 c.1026_1029del1G c.1155_1209del154	p.Pro342Glnfs * 43	Negative for mutations	Not done
10	c.1137 C>T c.1157_1200del144	p.Pro379Pro (SNP rs61752980) p.Leu386Glyfs * 4	Not done	Not done
11	c.488_995del508 c.1061_1062del1GC c.1229_1240del112	p.Gly163_Ser332del	Not done	Not done
12	c.401 C>G c.1168_1173del6	p.Ser134Cys p.Pro390_391del (SNPrs61753008)	Negative for mutations	Positive for c.1168_1173del6 p.Pro390_391del (SNPrs61753008)

Black: Represents mutations on different alleles. ■

Gray: Represents mutations on same alleles. ■

* For Participant number 1, another variation was detected: c.1180 G>A (p.Glu394Lys).

** For Participant number 2, another variation was detected: c.378-109 A>G. Participants 10, 11, and 12 were determined to be *in cis* from initial molecular testing. In terms of parental analysis for Participants 11 and 12, initial mutation analysis identified these mutations as *in cis* contiguous mutations and for this reason the genetic information of the parents was not obtained.

TABLE III
***MECP2* MUTATIONS: CONTIGUOUS**

Individuals with more than one contiguous nucleotide change in the *MECP2* coding sequence were identified. Similar to the parental analysis in Table II initial mutation analysis identified these mutations as *in cis* contiguous mutations.

Participant	Mutations cDNA level	Mutations Amino Acid level	Maternal Information	Paternal Information
1	c.1408_1411del4 c.1408_1411insTG	p.Asn470Trpfs X16	Not done	Not done
2	c.820_1153del334 c.820_1153ins67	Mutation does not disrupt reading frame, but yields protein with 89 fewer amino acids	Not done	Not done
3	c.855_859del4 c.855_859ins12	p.Ala287Lys fsX46	Not done	Not done

TABLE IV
CLINICAL SEVERITY

Two quantitative measures of clinical status, the Clinical Severity Scale (CSS) and the motor behavioral analysis (MBA) were completed for each participant seen on the Natural History Study. No differences were detected in the MBA and the CSS for those individuals with more than one mutation compared to those individuals with a single mutation. Participants with large frameshift or nonsense mutations (# 4, 6, 7, 8, 9, 10, 11, and 12) did not have differences in clinical severity compared to participants with two missense mutations or a missense mutation and a deletion of one amino acid (see results section).

Participant	Participant CSS Mean (range)	Database CSS Mean (SD) Range	Participant MBA Mean (range)	Database MBA Mean (SD) Range
From Table I				
1	25.8 (23-31)	16.8 (6.9) 6-35	55.3 (48-61)	41.0 (15.4) 3-79
2	29.0 (28-31)	17.0 (8.7) 1-34	57.0 (50-64)	39.3 (19.0) 1-86
3	26.5 (23-30)	25.3 (7.2) 10-43	51.5 (43-58)	52.0 (15.3) 27-96
4	14.3 (12-17)	20.9 (9.0) 1-43	32.9 (26-36)	45.2 (17.9) 1-87
5	18.2 (17-20)	17.0 (8.7) 1-34	28.8 (25-32)	39.3 (19.0) 1-86
6	14.3 (12-15)	17.0 (8.7) 1-34	39.5 (30-45)	39.3 (19.0) 1-86
7	25.6 (22-31)	17.0 (8.7) 1-34	53.8 (45-63)	39.3 (19.0) 1-86
8	23.8 (20-27)	17.0 (8.7) 1-34	51.8 (49-55)	39.3 (19.0) 1-86
9	21.3 (17-26)	25.5 (7.9) 6-41	35.5 (26-55)	53.7 (14.8) 18-85
10	12.0 (9-16)	16.2 (7.4) 1-35	28.0 (20-38)	37.9 (16.7) 1-79
11	31.9 (26-35)	25.3 (7.2) 10-43	56.1 (47-66)	52.0 (15.3) 27-96
12	17.9 (17-19)	25.5 (7.9) 6-41	32.0 (28-38)	53.7 (14.8) 18-85
From Table II				
1	31.0 (29-34)	17.0 (8.7) 1-34	61.4 (54-66)	39.3 (19.0) 1-86
2	19.7 (19-20)	25.5 (7.9) 6-41	37.7 (31-43)	53.7 (14.8) 18-85
3	18.2 (17-20)	25.5 (7.9) 6-41	35.5 (31-40)	53.7 (14.8) 18-85