Molecular analysis of MECP2 gene in Egyptian patients with Rett syndrome

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Abstract  Rett syndrome (RTT) is a progressive neurodevelopmental disorder that affects mainly females comprising one of the most common causes of mental retardation in females. Mutations in the X-linked MECP2 gene have been identified to be the major cause for RTT. This study represents one of the limited MECP2 molecular analyses done on Egyptian patients with RTT, in which direct sequencing of MECP2 coding region in 10 female Egyptian patients provisionally diagnosed to have RTT was carried out. Four different pathogenic mutations were identified in four patients; three missense (C380T, C397T and C916T) and one nonsense (C382T). The four mutations, C → T transitions, were located in exon four. Patients with MECP2 mutation showed the clinical course of
1. Introduction

Rett syndrome (RTT) (MIM 312750) is an X-linked dominant [1] neurodevelopmental disorder that affects mainly females [2] with a prevalence estimated to be 1 in each 10,000–15,000 female births [3,4]. RTT was first described by the Austrian pediatrician Andreas Rett in 1966 [5], however his article attracted little attention because it appeared in a German language journal that was not widely read out in Europe. In 1983, the Swedish researcher Bengt Hagberg published a report of 35 cases from Sweden, France and Portugal in Annals of Neurology which led to the worldwide recognition of RTT [2].

Girls with typical RTT are essentially characterized by normal birth and apparently normal psychomotor development during the first 6–18 months of life. The affected females then enter a short period of developmental stagnation followed by a period of rapid regression, during which they lose acquired speech and purposeful hand use, and showed acquired microcephaly, autistic features, and walking problems. The hallmark of the disease is the loss of purposeful hand use and its replacement with repetitive stereotyped hand movements. Secondary characteristics may include seizures, breathing abnormalities, vasomotor disturbances, skeletal deformities and abnormal muscle tone. By puberty, most patients stabilize and some may recover some skills [6]. In addition to the classic form of RTT, five atypical variants have been delineated on the basis of clinical criteria. Each variant lacks some of the necessary criteria of the classic form and can be milder or more severe. The milder variants are the preserved speech, the forme fruste and the late regression variants. The more severe forms are the early-seizure-onset and the congenital variants [7]. Most females with RTT survive to the middle age [8].

Approximately 99.5% of RTT cases are sporadic. In the few familial cases, the mutation is either present in the asymptomatic mother or due to germline mosaicism in one of the parents. The lack of phenotypic expression in the asymptomatic carrier mothers was shown to correlate with skewed X chromosome inactivation (XCI) pattern [9].

Numerous reports indicated that mutations in the coding sequence of MECP2 gene (the gene that encodes methyl-CpG-binding protein2, MeCP2) (MIM 300005) are the major cause of most typical cases of RTT. MECP2 mutations were also found, but less frequently, in girls with atypical forms [10]. MECP2 mutations were identified throughout the coding region of the gene including missense mutations, nonsense mutations, small insertions or deletions, splicing mutations, and large rearrangements (duplications or complex deletions) [11].

MECP2 is a four-exon gene located at the terminal end of the long arm of X chromosome (Xq28) [12]. It is ubiquitously expressed [13], however its high levels have been detected in the brain [14] where it is involved in the maturation of neurons [15]. Loss of MeCP2 functions in the brain leads to reduction in neuronal size and in the length and number of dendrites [16] and subsequently causing deficits in synaptic formation and/or transmission [17]. The assumption that MeCP2 is mainly required for the maturation of existing neurons rather than the development of new neurons from precursor cells may explain the delayed onset of RTT [18].

MeCP2 was thought to be a transcriptional repressor that prevents unscheduled transcription of other genes by binding to methylated CG dinucleotides in some gene promoters and recruiting histone deacetylases (HDACs), ultimately causing chromatin compaction and gene silencing [19]. MECP2 mutations result in apparent expression of other genes leading to RTT progression [20]. So far several genes associated with brain development were reported as MeCP2 targets such as brain-derived neurotrophic factor (BDNF) [21], DLX5 [22], glucocorticoid-regulated genes [23], the four ID genes [24], a transmembrane modulator of Na⁺, K⁺-ATPase activity (FXYD1) [25], and protocadherins PCDH11 and PCDH17 [26]. MeCP2 role may be more complex than it was thought, MeCP2 was suggested to be implicated in the formation of chromatin loop at the repressed loci [22], regulation of RNA splicing [27] and transcriptional activation of some genes [28].

MeCP2 contains two major functional domains; methyl-CpG-binding domain (MBD) [29] and transcriptional repression domain (TRD) [19,30]. Within the TRD, there is a nuclear localization signal (NLS) that mediates the transport of the protein into the nucleus [31].

This study has been carried out at the Human Stem Cell Lab, CEAS, and core genomic lab - NRC. PCR and direct sequencing were used to analyze the coding sequence of MECP2 in Egyptian patients with RTT.

2. Materials and methods

2.1. Subjects

Ten female patients, included in this study, were provisionally diagnosed to have RTT. They were identified at the Out-patients Clinic of the Clinical Human Genetics Department, National Research Centre.

2.2. Mutation analysis

DNA of RTT patients was extracted from peripheral blood leukocytes using the salting out protocol [32]. The three coding exons (exons 1, 3 and 4) and the flanking intronic sequences of MECP2 were amplified in overlapping fragments. Primers used for exons 3 and 4 were previously reported by Bienvenu et al. [33]. We have designed other set of primers to generate shorter fragments for sequencing.

PCR was performed in a total volume of 30 µl containing 100 ng of genomic DNA, 30 pmol of each primer, 200 µM of dATP, dCTP, dTTP and dGTP, 1.5 µM MgCl2, 1X Taq buffer and 2.5 U Taq polymerase (Fermentas, EU). The PCR products were purified using the PCR purification kit (Qiagen, Hilden, Germany) and sequenced with Big dye Terminator V3.1
cycle sequencing Kit (Applied Biosystems, California, USA) and ABI prism 310 Genetic Analyzer (Applied Biosystems, California, USA).

2.3. X-chromosome inactivation analysis

Patients with MECP2 mutation were tested for X chromosome inactivation pattern using the same protocol described previously by Calvo et al. [34].

3. Results

All patients were, basically, autistic, microcephalic and showed repetitive stereotypic hand movements. Therefore, they were referred with a provestional diagnosis of Rett syndrome and requesting MECP2 molecular analysis. The Clinical criteria of the studied patients are summarized in Table 1 and the score achieved by each patient according to RTT checklist described by Huppke et al. [35] is showed in Table 2. Molecular analysis of MECP2 gene in those patients revealed four different disease causing mutations in four unrelated patients (Table 3). The identified mutations located in exon 4; three were within the MBD and one in the TRD (Fig. 1). One novel silent mutation in exon 3 (C210T) was also reported in patient 1, in whom a pathogenic mutation (P127L) was detected (Fig. 2). Analysis of XCI pattern in patients with MECP2 mutations was informative in all of them and revealed random pattern in three patients and skewed pattern in only one patient (Fig. 3). Sequence analysis of MECP2 mutations in mothers of female patients with positive MECP2 mutation showed that they were negative for the mutation detected in their daughters.

4. Discussion

RTT is one of the most common causes of mental retardation in females with a prevalence estimated to be 1 in each 10,000:15,000 female births [3,4]. MECP2 gene mutations were identified to be the major cause of RTT. They are found in 80–90% of classic RTT patients and in 20–40% of patients with RTT variants [37]. Detection of the underlying cause in RTT patients will confirm the diagnosis, helping clinicians to manage their patients better and to offer precise counseling. Furthermore, it may provide insight regarding genotype-phenotype correlation.

This study represents one of the limited molecular analyses of MECP2 gene in Egyptian RTT patients with RTT. A previous report from our group was published in 2007 [38]. Direct sequencing of the MECP2 coding sequence of 10 female patients that included in this study revealed four different pathogenic mutations in four unrelated patients; three missense and one non sense. Generally, MECP2 mutations were detected in about 80% of RTT patients [39,40]. However, some previous studies showed relatively low rate of mutation detection and this mainly might depend on the clinical selection of the studied patients. Xiang et al. [41] screened the MECP2 gene for mutations by direct sequencing in 68 RTT cases and only a total of 27 patients (40%) were found to have mutations in the MECP2 gene. Raizis et al. [42] analyzed the MECP2 coding region by both direct automated DNA sequencing and MLPA in 74 patients with global developmental delay and mental retardation from New Zealand. The MeCP2 mutations among this selected group were only 20%.

The checklist for RTT described by Huppke et al. [35] seems to be effective in giving a better screening tool. According to this checklist, molecular analysis should be carried out only in patients achieving a score of 8 or more out of 12. In this study, patients with detected MECP2 mutations had a score of 10 at least. The studied patients were under continuous clinical follow up and it has been reported that the consanguineous parents of P7 have got recently another affected microcephalic daughter. In association with that, our index case P7 may be a case of autosomal recessive microcephalic disorder rather than RTT.

As RTT is an X-linked disorder, the X chromosome inactivation (XCI) pattern will have a significant impact on the clinical phenotype in patients with skewed XCI pattern where the disease severity decreases if the X chromosome with the normal gene is activated in majority of cells and vice versa. Analysis of the XCI pattern in our patients with positive MECP2 mutation revealed skewed pattern of inactivation in only one patient (P4) that couldn’t walk, speak and use hand. Other studies reported that R306C, the same mutation detected in this patient, might be generally associated with a relatively milder phenotype [43-46]. Therefore it can be postulated that in this patient, the XCI pattern favored activation of the X chromosome carrying the mutant allele in a large number of cells.

Generally, it was indicated that random XCI was the main pattern reported in RTT cases [47] denoting that XCI pattern is not a main modification factor on clinical phenotypes of RTT.

The three missense mutations identified in this study were P127L, R133C and R306C. P127L and R133C are located in the MBD, but R306C in the TRD. In previous reports, P127L showed low recurrence rate. To the best of our knowledge it was reported only 4 times before, the first report in 2001 in a patient with PSV [48]. Then, it was later identified in only 3 patients; 2 patients in a French study [49] and one patient in another study in China [9]. In contrast, R133C and R306C are of the most commonly occurring MECP2 mutations accounting for about 5.4% and 6.4% of RTT patients respectively [11]. Generally, it has been shown that the missense mutations were associated with milder phenotypes than truncating mutations [43,50]. Ham et al. [51] revised the mutations detected in 45 patients reported in 4 studies [52–55] with the milder RTT variants (PSV and forme fruste). They found that those patients mainly had carboxyl-terminal truncations and eight missense mutations. Of these missense mutations were P127L, R133C and R306C. The other five missense mutations identified were E10Q, T158 M, T158A, R168X and P302A. Hence, they inferred that a patient with a mild phenotype is likely to disclose either a carboxyl-terminal truncation or one of these missense mutations [51]. In our study, the mutations P127L, R133C and R306C were associated with classical course of RTT rather than a milder variant (Patients 1, 3 and 4).

Leonard et al., 2003 studied 24 patients having R133C to examine the phenotype associated with this mutation specifically and they found that the phenotype of a patient with R133C mutation is overall milder with better ambulation, hand use and a greater likelihood of being able to use speech [56]. Subsequently, Neul et al. [57] studied a large cohort of 245 patients with typical RTT. They identified R133C in 12 patients and reported that it was associated with relatively mild phenotype. Most patients with R133C preserved some hand
<table>
<thead>
<tr>
<th>Patient number and initial</th>
<th>Age at regression</th>
<th>Ability to walk</th>
<th>Ability to speak</th>
<th>Ability to use hand</th>
<th>Stereotypical hand movements</th>
<th>Postnatal microcephaly</th>
<th>Seizures</th>
<th>Behavioral abnormality</th>
<th>Muscle tone and reflexes</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1-WA</td>
<td>At 1 year</td>
<td>Walked with gait ataxia</td>
<td>Lost</td>
<td>No hand use</td>
<td>Clapping movements</td>
<td>47 cm (SD $-2.7$) at 4.5 years</td>
<td>Mild generalized epilepsy</td>
<td>Autistic features, swinging of moods, grinding</td>
<td>Hypotonia, brisk reflexes</td>
</tr>
<tr>
<td>P2-NW</td>
<td>At 1.5 years</td>
<td>Never walked</td>
<td>No speech</td>
<td>No hand use</td>
<td>Washing movements</td>
<td>47 cm (SD $-3.6$) at 8 years</td>
<td>Not reported in her file</td>
<td>Autistic features, pulls her clothes, grinding</td>
<td>Hypotonia, brisk reflexes</td>
</tr>
<tr>
<td>P3-NE</td>
<td>At 1.5 years</td>
<td>Weak walking</td>
<td>Lost</td>
<td>Impaired hand use</td>
<td>Abnormal hand movements</td>
<td>48.5 cm (SD $-2.6$) at 9 years</td>
<td>Frequent seizures</td>
<td>Generalized epilepsy</td>
<td>Autistic features</td>
</tr>
<tr>
<td>P4-AH</td>
<td>At 9 months</td>
<td>No walking</td>
<td>Lost</td>
<td>Lost</td>
<td>Clapping &amp; wringing movements</td>
<td>44 cm (SD $-3.8$) at 2.5 years</td>
<td>Not reported in her file</td>
<td>Autistic features, bruxism</td>
<td>Hypotonia, hyperreflexia, hypertonia, hyporeflexia, spasticity</td>
</tr>
<tr>
<td>P5- SW</td>
<td>Not reported</td>
<td>Started walking late (at 3 years)</td>
<td>No speech</td>
<td>No hand use</td>
<td>Flapping movements</td>
<td>44.7 cm (SD $-4.7$) at 4 years,</td>
<td>Not reported in her file</td>
<td>Autistic features, Laughing spells, babbling, grinding</td>
<td>Hypotonia, hyperreflexia</td>
</tr>
<tr>
<td>P6- SR</td>
<td>At 14 months</td>
<td>Started walking late (at 2 years)</td>
<td>No speech</td>
<td>Lost</td>
<td>Clapping movements</td>
<td>45.8 cm (SD $-2.5$) at 3 years</td>
<td>No seizures</td>
<td>Autistic features, babbling, spitting</td>
<td>Not reported</td>
</tr>
<tr>
<td>P7- SA</td>
<td>At early life</td>
<td>No walking</td>
<td>No speech</td>
<td>No hand use</td>
<td>Abnormal hand movements</td>
<td>43.5 cm (SD $-4$) at 4 years</td>
<td>Not reported in her file</td>
<td>Autistic features</td>
<td>Hypotonia, brisk reflexes</td>
</tr>
<tr>
<td>P8- NM</td>
<td>At 8 months</td>
<td>Impaired walking</td>
<td>Impaired speech</td>
<td>Impaired hand use</td>
<td>Abnormal hand movements</td>
<td>45 cm (SD $-3.5$) at 6 years</td>
<td>Generalized and tonic-clonic, epilepsy</td>
<td>Autistic features</td>
<td>Not reported</td>
</tr>
<tr>
<td>P9- WS</td>
<td>At 7 months</td>
<td>Impaired walking</td>
<td>Impaired speech</td>
<td>Impaired hand use</td>
<td>Abnormal hand movements</td>
<td>46 cm (SD $-2$) at 3 years</td>
<td>Epileptic fits</td>
<td>Autistic features, babbling</td>
<td>Hypotonia, brisk reflexes</td>
</tr>
<tr>
<td>P10- MS</td>
<td>At 9 months</td>
<td>Walked with aid</td>
<td>No speech</td>
<td>No hand use</td>
<td>Clapping &amp; wringing movements</td>
<td>45 cm at 3.5 years</td>
<td>Not reported in her file</td>
<td>Autistic features</td>
<td>Hypotonia, reflexes</td>
</tr>
</tbody>
</table>

Impaired walking, speech, hand use: skill ability is affected in a certain way.
Use (92%), a large percentage was able to walk alone (75%) and a significant proportion spoke words (50%). On the other hand, R306C was identified in another 21 patients and it was shown that a large set of patients with R306C could walk (67%) and retained some hand use (52%), but very few were able to use words. In our study, both patients with R133C and R306C showed lack of their ability to walk and use their hands, however, they showed different ambulation ability. While the patient with R133C could walk alone, the one with R306C didn’t walk. In the latter, this may be attributed to the skewed X chromosomes inactivation identified in this patient. Furthermore, it has been demonstrated that patients with R133C and R306C tend be associated with heightened anxiety and fear [58]. Several studies demonstrated that R133C strongly impaired MeCP2 binding to methylated DNA [59–61]. On the other hand, R306C probably affects the ability of MeCP2 to recruit corepressor complexes impairing its function in the process of transcriptional repression [62].

**Table 2** RTT checklist for studied female patients (the checklist is quoted from Huppke et al. [35]).

<table>
<thead>
<tr>
<th>Clinical criterion</th>
<th>Patients with MECP2 mutation</th>
<th>Patients without MECP2 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with MECP2 mutation</td>
<td>P1-WA</td>
<td>P2-NW</td>
</tr>
<tr>
<td>Normal prenatal and perinatal period</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Normal psychomotor development during the first 6 months</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Normal head circumference at birth</td>
<td>Not measured at birth, however the parents didn’t notice relatively small head circumference at birth</td>
<td></td>
</tr>
<tr>
<td>Deceleration of head growth</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Hand skills (1 if never, 2 if lost)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Stereotypic hand movements</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Communication dysfunction and social withdraw</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Acquired language (1 if never, 2 if lost)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Severe psychomotor retardation</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Impaired or absent locomotion</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Score</td>
<td>≥11</td>
<td>≥10</td>
</tr>
<tr>
<td>n.d.: Not documented.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3** Mutations of the MECP2 gene detected in the studied RTT patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Mutation type</th>
<th>Domain</th>
<th>Exon</th>
<th>X inactivation pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1-WA</td>
<td>C380T</td>
<td>P127L</td>
<td>Missense</td>
<td>MBD</td>
<td>Exon 4</td>
<td>Random</td>
</tr>
<tr>
<td>C210T*</td>
<td></td>
<td></td>
<td>Silent –</td>
<td>–</td>
<td>Exon 2</td>
<td></td>
</tr>
<tr>
<td>P2-NW</td>
<td>C382T</td>
<td>Q128X</td>
<td>Missense</td>
<td>MBD</td>
<td>Exon 4</td>
<td>Random</td>
</tr>
<tr>
<td>P3-NE</td>
<td>C397T</td>
<td>R133C</td>
<td>Nonsense</td>
<td>MBD</td>
<td>Exon 4</td>
<td>Random</td>
</tr>
<tr>
<td>P4-AH</td>
<td>C916T</td>
<td>R306C</td>
<td>Missense</td>
<td>TRD</td>
<td>Exon 4</td>
<td>Non-random</td>
</tr>
</tbody>
</table>

* this sequence variation has not been reported before.

![Figure 1](image-url) Distribution of the identified pathogenic mutations along the coding sequence of MECP2 gene. Diagramatic illustration of MECP2 gene and its function domains (this figure was adapted from Dragich et al. [36]). Nucleotides are numbered from the first nucleotide of the start ATG codon of the β isoform. The coding sequences for the MBD and TRD are indicated in yellow and red respectively. The NLS is hatched. Missense mutations are shown above and nonsense mutation below the sequence.
As P127L shows low recurrence rate, little information was reported about its associated clinical phenotype and its functional consequences on MeCP2. Our patient with this mutation is similar to that having R133C. She was also able to walk, but couldn’t speak or use hands.

Q128X, the nonsense mutation identified in our study is located in the MBD and presenting one of the few nonsense mutations identified in this domain. Most mutations reported in this domain were missense, however the nonsense mutations were clustered in the TRD domain and the region between the MBD and the TRD (the inter-domain (ID) region) [49,63]. Q128X has very low recurrence rate. To our knowledge, it was reported in only one patient in the study of Philippe et al., 2006, in which no clinical data were documented for the affected individual [49]. Previous reports documenting that truncating (nonsense and frameshift) mutations, in general, are associated with a more severe disease phenotype than missense mutations [43,50]. However, a milder disease was noted in patients with truncating mutations within or downstream of the TRD as compared with those who have truncating mutations upstream of the TRD. Dragich et al. [36] explained this difference in truncating mutations outcome as a consequence of their gene location. Huppke et al. [52] found that mutations lead to either a complete or partial truncation of the region coding for the nuclear localization signal (NLS) is associated with a more severe phenotype than other truncating mutations. They suggested that mutations leading to a truncation of the NLS produce proteins that will remain in the cytoplasm with more loss of protein function. However missense muta-

**Figure 2** Electropherograms of DNA sequencing for MECP2 mutations identified in RTT patients in this study. Substituted nucleotides are indicated by arrows and substituted amino acids are underlined. All sequences are in the sense orientation.

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and brisk reflexes. RTT such as vasomotor instability, constipation, hypotonia, respiratory dysfunction, this girl showed abnormal breathing. Truncating mutations have a higher incidence of the awake variant. However, she assessed a relatively high combined severity score for the abilities of hand use, speech and walk-
ing. She was unable to walk, speak or use hand. In accor-
dance with Amir et al. [64] who found that patients with truncating ones downstream of the NLS retain residual protein function. Our patient with Q128X showed a classical phenotype with normal development at the first months of life and no seizures in early life denoting that she is not a case of congenital or early onset seizures RTT variant. However, she assessed a relatively high combined severity score for the abilities of hand use, speech and walking. She was unable to walk, speak or use hand. In accordance with Amir et al. [64] who found that patients with truncating mutations have a higher incidence of the awake respiratory dysfunction, this girl showed abnormal breathing. Additionally, this patient exhibited other minor criteria of RTT such as vasomotor instability, constipation, hypotonia and brisk reflexes.

All detected mutations including the silent one are C → T transitions. However in P127L and Q128X, the transitioned cytosines are not at CpG dinucleotides. Hence, these 2 mutations may be resulted from transitions of unmethylated cytosines which are less amenable for chemical modification and this may explain the relatively low recurrence rate of both of them.

Although Approximately 99.5% of RTT mutations arise de novo, mothers may rarely carry the mutation without manifesting the phenotypic expression due to skewed XCI. We investigated the mutation presence in mothers of patients with positive MECP2 from whom DNA could be obtained indicating that no mutation was present in the maternal DNA.

RTT occurs in all ethnic groups across the world [65]. However, no discrimination in the spectrum and the frequency of MECP2 mutations could be revealed among the different populations. This can be explained as most MECP2 mutations originate de novo [66]. Molecular analysis of MECP2 gene in 7 Tunisian patients with RTT identified T158M mutation in 4 patients (more than 50%) [62], however this mutation was not detected in our studied patients.

5. Conclusion and recommendations

This is one of the limited genetic studies of Rett syndrome in Egypt. The relatively low observed frequency of MECP2 mutations may reflect a wide spectrum of mental disability disorders. However, analysis of large rearrangements should be carried out in patients without MECP2 mutation to reduce the risk of false negatives and to increase the sensitivity.

References


Figure 3 Analysis of X chromosome inactivation (XCI) pattern using the androgen receptor methylation assay. Ethidium bromide stained agarose gel electrophoresis for PCR products of patients with identified MECP2 mutations. Aliquot of DNA of peripheral blood leukocyte from each patient was used directly as a template (+ lanes) and another aliquot was digested with HpaII prior to PCR amplification (+ lanes). All analyzed patients are informative i.e, have alleles with different size repeats. Patient 4 is the only one that had non random. R: random - NR: non random.


