Araştırma Makalesi
Research Article

Clinical evaluation of patients with classical Rett Syndrome and MECP2 gene analysis

KLASİK RETT SENDROMU OLAN HASTALARIN KLİNİK DEĞERLENDİRMESİ VE MECP2 GEN ANALİZİ

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ABSTRACT

Objective: This retrospective study aims to evaluate the clinical manifestations in genetically confirmed classical Rett syndrome (RTT) patients.

Materials and Methods: Sixteen patients clinically diagnosed with classical RTT between 2010 and 2020 were evaluated according to diagnostic criteria for this syndrome. The results of pyrosequencing, Sanger sequencing, and multiplex ligation-dependent probe amplification of MECP2 gene were inspected.

Results: Besides the four main criteria of RTT which are necessary for the diagnosis of classical RTT, diurnal bruxism (16/16 patients), epilepsy (11/16; 68.7%) and microcephaly (11/16; 68.7%) were the most common features in the patients. Early onset epilepsy was detected in six patients (6/11; 54.5%). Four of them had drug-resistant epilepsy. Four out of 5 patients with late onset epilepsy had a good response to anticonvulsant treatment. Scoliosis, breathing problems, peripheral vasomotor disturbances and sleep problems were detected in 37.5%, 18.7%, 25% and 12.5% of the patients, respectively. Nine different MECP2 gene variants were identified in 16 patients. Nonsense variant was the most common variant type (7/16; 43.7%). The detected variants were p.Arg168Ter (n=5); p.T158M (n= 2); p.R255X (n= 2); p.Arg106Trp (n=1); p.Arg270GlufsTer19(n=1);p.Lys286ProfsTer2 (n=1); p.Leu386_Thr400delinsPro (n=1); Exon 4 del (n=2) and Exon 3-4 del (n= 1).

Conclusion: The p.Arg168Ter variant is common in classical RTT patients in Turkey. Diurnal bruxism, microcephaly and epilepsy are common features in this syndrome. Epilepsy develops in more than half of the patients with classical RTT and especially early-onset epilepsy tends to be drug resistant in a substantial proportion of the patients.

Keywords: Rett Syndrome, MECP2 gene, epilepsy, diurnal bruxism, microcephaly, drug resistant

ÖZ

Amaç: Bu retrospektif çalışma, genetik tanı almış klasik Rett Sendromu (RTT) hastalarında, klinik bulguları değerlendirmeyi amaçlamaktadır.

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Gereç ve Yöntem: 2010-2020 yılları arasında, genetik polikliniğinde RTT tanısı almış 16 hasta, sendromun tanı kriterlerine göre değerlendirildi. Pyrosekans, Sanger sekans ve multipleks ligasyona bağlı prob amplifikasyonu analiz sonuçları incelendi.

Bulgular: Klasik RTT tanısı için gerekli olan 4 ana kritere ek olarak, hastalarda en sık saptanan bulgular; diurnal bruksizm (16/16; %100), epilepsi (11/16; %68,7) ve mikrosefali (11/16; %68,7) idi. Erken başlangıçlı epilepsi, 6 hastada saptandı (6/11; %54,5). Bu hastaların dördünde, ilaca dirençli epilepsi vardı. Geç başlangıçlı epilepsisi olan 5 hastanın 4'ü antikonvülsan tedaviye iyi yanıt verdi. Skolyoz, nefes alma problemleri, periferik vazomotor bozukluk ve uyku problemleri sırasıyla %37,5; %18,7; %25 ve %12,5 sıklıkta saptandı. 16 hastada, toplam 9 farklı MECP2 gen varyantı tanımlandı. Nonsense varyant, en sık varyant tipiydi (7/16 hasta; %43,7). Çalışmada saptanan varyantlar; p.Arg168Ter (n=5); p.Thr158Met (n=2); p.Arg255Ter (n=2); p.Arg106Trp (n=1); p.Arg270GlufsTer19 (n=1); p.Lys286ProfsTer2 (n=1); p.Leu386_Thr400delinsPro (n = 1); Exon 4 del (n=2) ve Exon 3-4 del (n=1) idi.

Sonuç: p.Arg168Ter, patojenik varyantı, Türk klasik RTT hastalarında yaygındır. Diurnal bruksizm, mikrosefali ve epilepsi, bu sendromun sık bulgularındandır. Klasik RTT'li hastaların yarısından fazlasında epilepsi gelişir ve özellikle erken başlangıçlı epilepsi, hastaların önemli bir kısmında ilaca dirençli olma eğilimindedir.

Anahtar Sözcükler: Rett Sendromu, MECP2 geni, epilepsi, Diurnal bruksizm, mikrosefali, ilaç direnci

Rett syndrome (RTT; MIM #312750) is an early-onset neurological disorder, and is among leading genetic reasons for intellectual disability in female gender with a prevalence of 1:10000-15000 in female live births (1). There are two major clinical forms of RTT: 1- classical or typical, 2-variant or atypical (2). It is based on clinical evaluation with diagnostic criteria first described in 2002 (3). A revised diagnostic criterion have been reported in recent years to simplify the diagnosis of classical and atypical RTT (4). To diagnose classical RTT, history of regression should be present and the patients should have all of the main criteria and not have any exclusion criteria (Table 1). Minimum two of the main criteria and five of the eleven supportive criteria should be present in the patient for atypical RTT diagnosis.

RTT is an X-linked dominant disorder, with the most of the patients being caused by mutations or deletions in MECP2 gene. This gene is located on Xq28 and is coding for the methyl-CPG-binding protein 2 (5). The MECP2 gene is comprised of four exons and the protein contains five main domains: The N-terminal domain (NTD); a methyl-CpG-binding domain (MBD); a transcriptional repression domain (TRD); the C-terminal domain (CTD) and an interdomain (ID) (6). The protein has two isoforms (MeCP2_e1 and MeCP2_e2). These proteins differ in N termini and relative expression levels in tissues. In the brain, MeCP2_e1 is the predominant isoform, and MeCP2_e2 is abundant in the liver, skeletal muscles and placenta (7, 8).

There is a high correlation between the mutations in MECP2 gene and RTT, particularly with its classical form, although they have been also detected in a small proportion of patients with intellectual disabilities and autism spectrum disorders (4-9, 11). About 800 different variants of MECP2 gene, including point mutations, insertions, duplications, small or large deletions, have been described (12). MECP2 gene mutations are responsible for 95-97% of classic and about 50-70% of atypical RTT (13, 14). The most common pathogenic variants in MECP2 gene are p.Arg106Trp, p.Arg133Cys, p.Arg306Cys, p.Thr158Met, p.Arg168Ter, p.Arg255Ter, p.Arg270Ter and p.Arg294Ter. These variants are responsible for nearly 50% of the patients with RTT (15). In this study, we aimed to evaluate the clinical manifestations in 16 genetically confirmed classical RTT patients.

Table 1. The Revised diagnostic criteria for RTT. Modified from Neul JL et al: Ann Neurol 2010;68:944-950.

Revised diagnostic criteria for RTT 2010 **Consider diagnosis when postnatal deceleration of head growth observed**		
Required for typical RTT	1 A period of regression followed by recovery or stabilization 2 All main criteria and all exclusion criteria 3 Supportive criteria are not required, although often present in typical RTT	
Required for atypical RTT	1 A period of regression followed by recovery or stabilization 2 At least 2 out of the 4 main criteria 3 5 out of 11 supportive criteria	
Main Criteria	1 Partial or complete loss of acquired purposeful hand skills 2 Partial or complete loss of acquired spoken language 3 Gait abnormalities: Impaired (dyspraxic) or absence of ability 4 Stereotypic hand movements such as hand wringing/squeezing, clapping/tapping, mouthing and washing/rubbing automatisms	
Exclusion Criteria for typical RTT	1 Brain injury secondary to trauma (peri- or postnatally), neurometabolic disease, or severe infection that causes neurological problems 2 Grossly abnormal psychomotor development in first 6 months of life	
Supportive Criteria for atypical RTT	1 Breathing disturbances when awake 2 Bruxism when awake 3 Impaired sleep pattern 4 Abnormal muscle tone 5 Peripheral vasomotor disturbances	6 Scoliosis/kyphosis 7 Growth retardation 8 Small cold hands and feet 9 Inappropriate laughing/screaming spells 10 Diminished response to pain 11 Intense eye communication “eye pointing”

MATERIAL and METHODS

Patient Selection

16 female patients diagnosed with classical RTT according to the international criteria of RTT were included in the present study. Four patients were assessed at the Department of Medical Genetics, Pediatric Genetics and Pediatric Neurology of Behçet Uz Child Disease and Pediatric Surgery Training and Research Hospital, Izmir, Turkey. MECP2 gene screening of the patients was carried out by three different methods: pyrosequencing analysis, Sanger sequencing and MLPA. As a standard approach in our department, pyrosequencing analysis is used to detect eight common pathogenic variants including p.Arg106Trp, p.Arg133Cys, p.Arg306Cys, p.Thr158Met, p.Arg168Ter, p.Arg255Ter, p.Arg270Ter and p.Arg294Ter in patients clinically diagnosed with RTT. In patients with a normal pyrosequencing analysis, further Sanger sequencing of all coding exons of MECP2 is used. However, if the result is normal, MECP2 MLPA analysis is done.

Clinical manifestations and molecular results were collected from the medical records of the patients. Informed written consents were taken from the parents of all participants. Ethical approval for this study was obtained by the institutional review board at Dr. Behcet Uz Children’s Hospital, Izmir, Turkey.

Molecular Analysis

DNA isolation

Peripheral blood samples of 16 classical RTT patients were collected in EDTA tubes. Genomic DNA from the peripheral blood lymphocytes of all individuals was

extracted with QIAamp DNA mini kit (Qiagen, GMBH, Hilden, Germany) following the manufacturer protocol.

Pyrosequencing analysis

Eight target sequences encompassing p.Arg106Trp, p.Arg133Cys, p.Arg306Cys, p.Thr158Met, p.Arg168Ter, p.Arg255Ter, p.Arg270Ter and p.Arg294Ter pathogenic variants of MECP2 gene were amplified with PCR and sequenced with the pyrosequencing method (MECP2; ENST00000303391.6). PCR was performed using the PyroMark PCR Kit (Qiagen, Maryland, Germany). After the activation (15 min at 95°C), the DNA was amplified for 45 cycles under the following conditions: 30 secs at 94°C, 30 sec at 60°C and 30 sec at 72°C. Before pyrosequencing, PCR product was biotinylated binding to Streptavidin-coated sepharose beads captured with a vacuum tool. Once they were washed and denatured, single-stranded DNA was generated. This template DNA was released into the Pyrosequencing reaction plate. This plate included the sequencing primers. After primer annealing, this plate was inserted into the PyroMark instrument. Pyrosequencing reaction was conducted on the PyroMark Q24 instrument following manufacturer's instructions (PyroMark, Qiagen, Germany). PyroMark Q24MDx Software was used for the analysis of results.

Sanger sequencing analysis

All the coding exons and exon–intron boundaries of MECP2 gene were amplified using previously reported primers (16). The PCR products were purified and sequenced on an ABI PRISM 3500 automated DNA sequencer (Applied Biosystems). Each variation was confirmed by bidirectional sequencing. The standards and guidelines established by the American College of Medical Genetics were used for the classification of variants.

MLPA (Multiplex Ligation-dependent Probe Amplification) analysis

Mutation-negative samples were tested for large deletions with SALSA MLPA P015 probe mix (MRC-Holland, Amsterdam, and The Netherlands) following manufacturer's instructions. The MLPA products were quantified with capillary electrophoresis and ABI PRISM® 3500xl (Applied Biosystems®, Invitrogen Life Technologies, Carlsbad, CA, USA). Gene Mapper (Applied Biosystems, Foster City, CA, USA), and Coffalyser Software (MRCHolland) was used for MLPA analysis.

RESULTS

MECP2 gene analyzes and clinical findings of the 16 patients with classical RTT were evaluated (Table 2, 3). The median age was 3.7 years (range; 1-12 years). The detailed clinical features of the patients with MECP2 pathogenic variants are presented in Table 3. Besides the four main criteria of RTT, diurnal bruxism (16/16 patients; 100%;), microcephaly (11/16 patients; 68.7%) and epilepsy (11/16 patients; 68.7%) were the most common features (Table 3). When we evaluated the patients with epilepsy according to drug response, five out of them had drug-resistant epilepsy (5/11 patients; 45.4%). Early onset epilepsy which started before the age of three was detected in six patients (6/11patients; 54.5%). Two of these six patients had also a good response to the anticonvulsant treatment. In five patients, seizures were seen in 3 years of age or older (5/11; 45.4%). Only one of them had drug-resistant epilepsy.

Seven different MECP2 gene variants were identified in 13 patients by both sequence analyses (pyrosequence or Sanger sequence) (81.2%). All identified variants are present in the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/). Nonsense variant was the most common variant type with two different variants in seven patients (7/16 patients; 43.7%). The other identified variants were two missenses, two frameshifts and one insertion-deletion. The mutations that occur more frequently were p.Arg168Ter (n =5); p.T158M (n = 2) and p.R255X (n = 2) (Figure 1). Heterozygous exon 4 deletion (n = 2) and exon 3-4 deletion (n = 1) were detected by MLPA analysis (18.7%) (Table 2).

When we evaluate the clinical characteristics of the five patients with the most common pathogenic variant (p.Arg168Ter), in addition to the main criteria, microcephaly and diurnal bruxism were noted in all of them. While epilepsy, inappropriate laughing and screaming spells were noted in four patients, the other features were less common (Table 3).

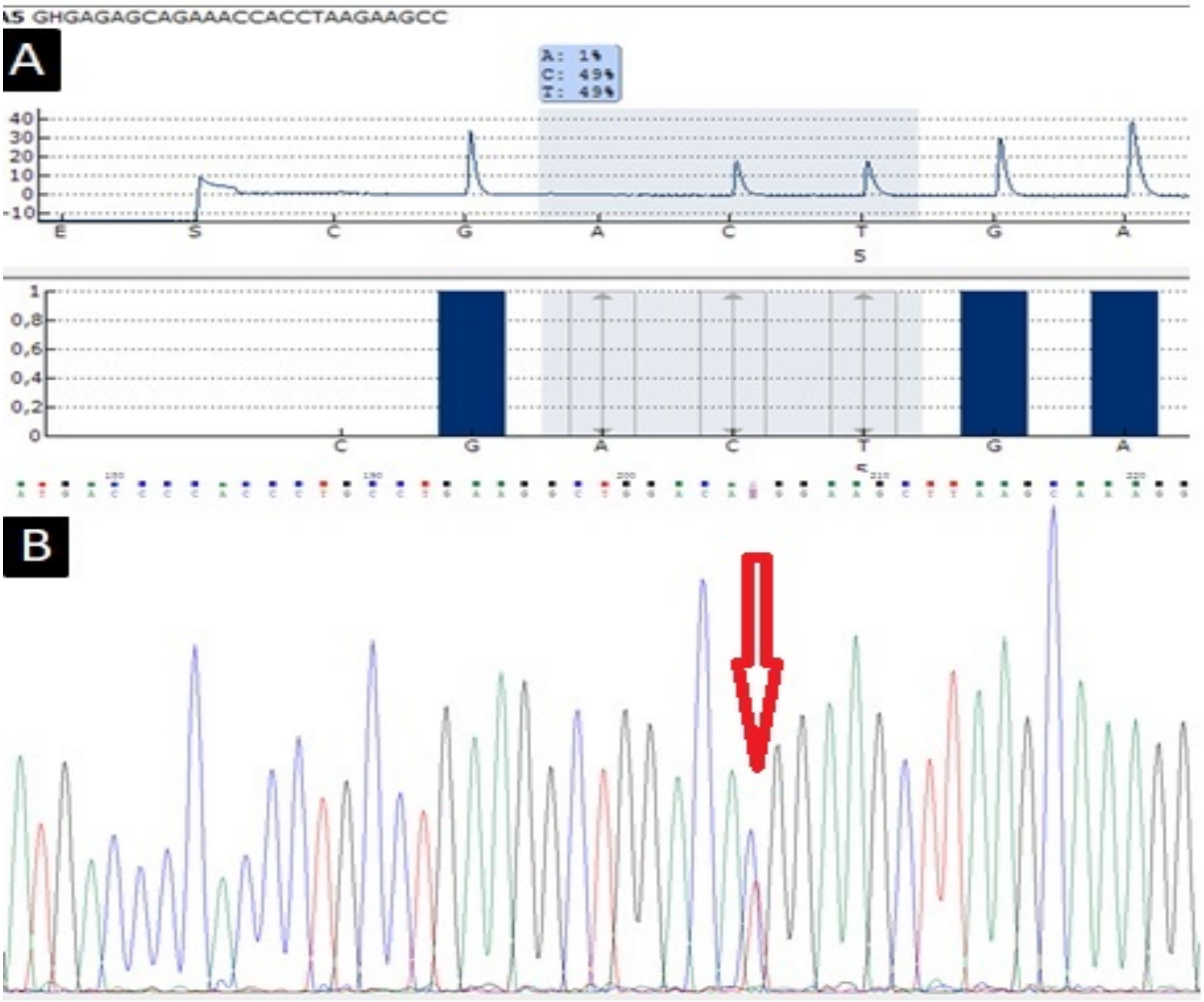

Figure 1. a-b: The Pyro and Sanger sequencing results of different patients (DAP, ×200).

A: Pyrosequencing data shows a heterozygous c.502C>T (p.Arg168Ter) variant, B: Sanger sequencing result of c.316C>T (p.Arg106Trp) variant.

Table 2. Identified pathogenic variants of MECP2 gene

Patients	Age of RTT Diagnosis	Mutation in cDNA	Mutation in amino acid	Exon	Domain	Method of molecular analysis
P1	2	c.316C>T	p.Arg106Trp	3	MBD	Pyrosequencing
P2	4	c.473C>T	p.Thr158Met	4	MBD	Pyrosequencing
P3	5	c.473C>T	p.Thr158Met	4	MBD	Pyrosequencing
P4	2,5	c.502C>T	p.Arg168Ter	4	ID	Pyrosequencing
P5	1,5	c.502C>T	p.Arg168Ter	4	ID	Pyrosequencing
P6	12	c.502C>T	p.Arg168Ter	4	ID	Pyrosequencing
P7	2,5	c.502C>T	p.Arg168Ter	4	ID	Pyrosequencing
P8	4	c.502C>T	p.Arg168Ter	4	ID	Pyrosequencing
P9	5	c.763C>T	p.Arg255Ter	4	TRD	Pyrosequencing
P10	1,5	c.763C>T	p.Arg255Ter	4	TRD	Pyrosequencing
P11	2	c.808delC	p.Arg270GlufsTer19	4	TRD	Pyrosequencing
P12	4,5	c.856_859delAAAG	p.Lys286ProfsTer2	4	TRD	Sanger sequencing
P13	5	c.1157_1198del	p.Leu386_Thr400delinsPro	4	CTD	Sanger sequencing
P14	4	Exon 4 del		4		MLPA
P15	2	Exon 4 del		4		MLPA
P16	3	Exon 3-4 del		3-4		MLPA

MBD: methyl-CpG binding protein, TRD: transcription repression domain, ID: intervening domain

Table 3: Clinical manifestations of the classical RTT patients with *MECP2* variants

Clinical Features	Patients with MECP2 mutations N: 16 (%)	Patients with p.Arg168Ter N:5	Patients with p.Thr158Met N:2	Patients with p.Arg255Ter N:2	Patients with ekzon 4 del N:2	Patients with p.Arg270Glufs*19 N:1	Patient with p.Arg106Trp N:1	Patient with c.856_859delAAAG N:1	Patient with c.1157_1198del N:1	Patient with ekzon 3-4 del N:1
Loss of acquired purposeful hand skills	16 (100%)	5	2	2	2	1	1	1	1	1
Loss of acquired spoken language	16 (100%)	5	2	2	2	1	1	1	1	1
Stereotypic hand movements	16 (100%)	5	2	2	2	1	1	1	1	1
Gait abnormalities	16 (100%)	5	2	2	2	1	1	1	1	1
Bruxism when awake	16 (100%)	5	2	2	2	1	1	1	1	1
Microcephaly	11(68.7%)	5	1	-	2	1	-	-	1	1
Epilepsy	11(68.7%)	4	2	1	2	-	-	1	1	-
Abnormal muscle tone	9 (56.2%)	2	2	-	2	1	-	-	1	1
Small cold hands and feet	6 (37.5%)	2	-	1	-	1	-	-	1	1
Inappropriate laughing/screaming spells	6 (37.5%)	4	-	1	-	-	-	-	-	1
Scoliosis or kyphosis	6 (37.5%)	2	1	1	-	1	-	-	1	-
Growth retardation	4 (25%)	1	-	-	1	1	-	-	1	-
Breathing disturbances when awake	3 (18.7%)	1	-	-	-	1	-	-	1	-
Intense eye communication - "eye pointing"	4 (25%)	1	-	1	1	-	-	-	1	-
Peripheral vasomotor disturbances	4 (25%)	1	-	-	-	1	-	-	1	1
Diminished response to pain	2 (12.5%)	1	-	-	-	-	-	-	1	-
Impaired sleep pattern	2 (12.5%)	-	-	-	-	-	1	-	-	1

DISCUSSION

In this study, we reported the results of the genetic analysis of MECP2 gene and clinical features in Turkish classical RTT girls. RTT syndrome caused by mutations in MECP2 gene is one of the most frequent reasons of mental retardation in females (5). DNA sequencing identifies pathogenic variants of MECP2 gene in about 80% of classic RTT patients (17). The eight most common pathogenic variants including p.Thr158Met, p p.Arg168Ter, p.Arg255Ter, p.Arg270Ter, p.Arg306Cys, p.Arg294Ter, p.Arg133Cys and p.Arg106Trp account for 47% of all the variants in MECP2 gene (15). Deletions including one or more exons of MECP2 gene were reported in approximately 10% of suspected RTT patients including classic, atypical, and unclassified (17-19). However, the frequency of large exon deletions increases up to 28% in classic RTT (19). There is only one previous study, which investigated MECP2 variants in Turkish RTT patients. In that study, about half of the patients (7/16) had missense mutations. Moreover, p.Arg106Trp (3 patients) and p.Arg168Ter (3 patients) variants were reported as the most common variants. Additionally 7 out of 8 common mutations of MECP2 gene were detected in that study. Large deletion was reported in only one patient (20). In our study, we detected seven different pathogenic variants in 81.2% of the patients with both sequence analyses and this rate was compatible with the literature. The most common type of variant was nonsense change (7/16 patients; 43.7%). Missense mutations were noted in only three patients (18.7%). While the most common pathogenic variant was p.Arg168Ter, consistent with previous data, p.Arg106Trp was detected in only one patient. Two large deletion were detected in three patients. The p.Arg294Ter pathogenic variant was not detected in our study and in the other study conducted in Turkey (20). The reason for this can be that p.Arg294Ter may not be an MECP2 variant often seen in Turkey. Approximately, 70% of the pathogenic MECP2 gene variants have been described in TRD and MBD domains, which are located in the 3rd and 4th exons. Most of the mutations are in the form of nucleotide transition from cytosine to thymine (C>T) and this is consistent with the possibility of hypermutability at methylated CpG dinucleotides (5, 21). In our study, All of the variants, except for deletions, had the same nucleotide transition (C>T) and were located in exon 3 and 4. Additionally, more than half of the variants were located in ID and TRD domains of MECP2 gene, which were also consistent with the literature.

There is a high variability in the clinical features of RTT. Amir et al. evaluated 78 classical RTT patients, and detected the relationship between truncating mutations and breathing abnormalities (22). Additionally, they reported that scoliosis was common in patients with missense mutations. Similarly, in our study, breathing abnormalities were detected in two patients with truncating mutations. Scoliosis was detected in six patients. Unlike the current literature, we found missense mutation in only one of them. The remaining five patients had nonsense (three patients) and frameshift (two patients) mutations. Diurnal bruxism, which was one of the supportive criteria for atypical RTT, was reported in 68 to 84 % of RTT patients (23, 24). Bebbington et al. reviewed 346 RTT patients with MECP2 variants and they have reported that common features of this syndrome including scoliosis, breathing problems, peripheral circulation problems and impaired sleep pattern were detected in 54.6%, 64.2%, 71.3% and 67.3% of RTT patients, respectively.26 In this study the mean age of the participants was > 9 year of age and they were followed-up till adulthood. No significant relationships between these features and mutation types were found (25). In our study, besides major diagnostic criteria, diurnal bruxism was the most common feature, which was detected in all classical RTT patients. However, we noted scoliosis, breathing problems, peripheral vasomotor disturbances and sleep problems in 37.5%, 18.7%, 25% and 12.5% of the patients, respectively. The rates of these clinical features in our study were lower than those reported by Bebbington et al., it may be associated with the lower mean age of the participants (25).

The prevalence epilepsy was reported as 58 to 80% in RTT patients (25-27). Seizures started around four years of age in about half of RTT patients (28, 29). The results of studies investigating the relationship between epilepsy and mutation are conflicting. Pintuati et al. reported that epilepsy was commonly seen in patients with large gene

deletions and p.Arg294Ter variant of MECP2 gene. Patients with p.Arg168Ter and p.Arg255Ter variants started having seizures earlier than patients with large gene deletions (30). However, Jian et al. evaluated 288 patients and they reported that seizure rates were found low in patients with p.Arg294Ter and p.Arg255Ter variants (29). Similarly Glaze et al., revealed 493 patients with MECP2 variants and found lower rates of epilepsy in patients with p.Arg255Ter variant (31). Nevertheless, Halbach et al. evaluated 137 RTT patients and no relationship was found between epilepsy and mutation types (26). In that study, a history of epilepsy was reported in 61% of the RTT patients, but in only 1 patient, seizures started before 4 years of age. In 18% of the females, epilepsy was uncontrolled or poorly controlled by medication. In the present study, the rate of epilepsy (68.75%) was compatible with the literature. However, the frequency of early onset epilepsy (54.5%) and the incidence of epilepsy around four years of age (9/11 patients; 81.8%) were higher than the current literature (26, 28). Additionally, resistant epilepsy (5/11 patients; 45.4%) was more commonly detected when compared with the study authored by Halbach et al. (26). Nissenkorn et al. reported that late-onset epilepsy is a good prognostic factor for the disease (32). In our study, four out of five patients with late-onset epilepsy had a good response to the anticonvulsant treatment. In accordance with Pintuati et al. (2010), epilepsy was detected in two patients with large deletion. Four of the five patients with p.Arg168Ter had epilepsy, but there was no difference between the patients regarding the age of onset of epilepsy. Consequently, we could not find any relationship between MECP2 variants and the age of onset of epilepsy.

In conclusion, the p.Arg168Ter variant is common in classical RTT patients in Turkey. Besides the four main criteria of RTT, which are necessary for the diagnosis of classical RTT, diurnal bruxism, microcephaly and epilepsy are common features in this syndrome. Epilepsy, which can be treated using anti-epileptic medications, develops in more than half of patients with RTT and especially early-onset epilepsy tends to be drug resistant in a substantial proportion of the patients.

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