ANALYSIS OF *MECP2* GENE MUTATIONS IN RETT SYNDROME PATIENTS

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By

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August, 2001

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ABSTRACT

ANALYSIS OF *MECP2* GENE MUTATIONS IN TURKISH RETT SYNDROME PATIENTS

Ayça Sayı
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Rett Syndrome (RTT) is a progressive X-linked dominant childhood neurodevelopmental disorder, affecting 1/10,000-15,000 girls. The disease-causing gene was identified as *MECP2* on chromosome Xq28, and mutations have been found in approximately 80% of patients diagnosed with RTT. We screened for eight recurrent *MECP2* mutations (R106W, P152R, T158M, R306C, R168X), one rare mutation (F155S) and one polymorphism (E397K) in 63 RTT patients divided into four groups as classic-RTT (n=43), variant-RTT (n=14), male-RTT (n=4), and familial-RTT (n=2). We identified the recurrent mutations in 18 cases. These are three R106W, two P152R, five T158M, five R306C, and three R270X mutations. R168X and F155S were not detected in our patients. Only one patient had the E397K polymorphism who also had the R306C mutation. All these mutations were confirmed via sequencing analysis. In exon 4 of *MECP2*, several deletion types of mutations are known. By PCR analysis, two patients were found to have an approximately 44 bp deletion in exon 4. Also, a novel mutation – T197M– was identified in one of the patients. We identified a boy affected by RTT who is mosaic for the R270X mutation, and had a normal male karyotype. This result show that a recurrent *MECP2* mutation could lead to a similar phenotype in females and males, if the male is a mosaic for the mutation in his somatic cells. *MECP2* mutation frequency for the four groups is as follows: 37.2% for the classic-RTT, 28.57% for the variant-RTT, and 25% for the male-RTT groups. No mutation was found in the familial group. We could not find a consistent correlation between the clinical symptoms and the type of mutations or the X chromosome inactivation patterns of the patients.

ÖZET

TÜRK RETT SENDROMU HASTALARINDA *MECP2* GENİNDEKİ MUTASYONLARIN ANALİZİ

Ayça Sayı
Moleküler Biyoloji ve Genetik Yüksek Lisans
Tez Yöneticisi: Doç. Dr. Tayfun Özçelik
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Rett Sendromu (RTT) X'e bağlı dominant kalıtım gösteren, ilerleyici özellikte, bir çocukluk dönemi nöral gelişim hastalığıdır. Büyük bir çoğunlukla kızları etkiler ve 1/10,000-15,000 sıklıkla görülür. Hastalığa neden olan genin Xq28'de bulunan *MECP2* olduğu tanımlanmıştır. RTT tanısı konan hastaların yaklaşık %80'inde bu genin mutasyonu bulunmuştur. Biz 63 RTT hastasını klasik-RTT (n=43), RTT-varyant (n=14), erkek-RTT (n=4) ve ailesel-RTT (n=2) olmak üzere dört gruba ayırdık ve bu hastalarda sekiz adet sık gözüken mutasyonu (R106W, P152R, T158M, R306C, R168X), bir adet nadir gözüken mutasyonu (F155S), ve bir adet polimorfizmi (E397K) taradık. Üç hastada R106W, iki hastada P152R, beş hastada T158M, beş hastada R306C ve üç hastada R270X mutasyonu olmak üzere toplam onsekiz sık gözüken mutasyonu saptadık. Bizim hastalarımızda R168X ve F155S mutasyonları bulunamamıştır. R306C mutasyonu olan bir hastada aynı zamanda E397K polimorfizmi saptanmıştır. Tüm bu mutasyonların varlığı sekans analizi ile doğrulandı. *MECP2*'nin dördüncü eksonunda bilinen birkaç delesyon tipi mutasyon bulunmaktadır. PCR analizi ile iki hastanın ekson 4'ünde yaklaşık 44 baz çifti delesyon bulundu. Hastaların birinde yeni bir mutasyon –T197M- saptanmıştır. Normal karyotipe sahip bir erkek RTT hastasında R270X mutasyonu saptadık. Bu mutasyon normal *MECP2* dizisi ile birlikte görüldüğü için hastada somatik mozaisizm olduğu sonucuna varılmıştır. Dört grup RTT hastası için *MECP2* mutasyon sıklığı sırasıyla: klasik-RTT için %37.2, RTT-varyant için %28.57, ve erkek-RTT grubu için %25 olarak bulunmuştur. Mutasyon tipi ile klinik semptomlar veya X kromozom inaktivasyon profilleri arasında anlamlı bir ilişki bulamadık.

TO MY PARENTS

BİRSEN , FEVZİ SAYI

AND

TO MY SISTER

NİLAY SAYI

FOR THEIR LOVE AND SUPPORT

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ABBREVIATIONS

APS	ammonium persulfate
ATP	adenine triphosphate
Bisacrylamide	N, N, methylene bis-acrylamide
bp	base pair
CpG	Cytosine guanine pair
cDNA	complementary DNA
dATP	adenosine deoxyribonucleoside triphosphate
dCTP	cytosine deoxyribonucleoside triphosphate
del	deletion
ddH_2O	deionized water
dGTP	guanosine deoxyribonucleoside triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
dTTP	tymine deoxyribonucleoside triphosphate
EDTA	ethylenediaminetetra-acetic acid
EtBr	ethidium bromide
EtOH	ethanol
g	gram
kb	kilobase
M	molar
min	minute
ml	milliliter
mM	millimolar
µl	microliter
NaOAc	sodium acetate
ng	nanogram
nm	nanometer
nt	nucleotide
ODA	optic density

PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pmol	picomol
rpm	revolution per minute
RTT	Rett syndrome
SDS	sodium dodecyl sulphate
sec	second
TBE	Tris, Boric acid, EDTA
TEMED	N, N, N, N-tetramethyl-1, 2 diaminoethane
U	unit
UV	ultraviolet
V	volt
μg	microgram
μl	microliter

I. Introduction

1.1. Rett Syndrome

Rett Syndrome (RTT) is an X-linked dominant neurodevelopmental disorder and the second most common cause, after Down syndrome, of severe mental retardation in females (Christodoulou *et al.*, 2001). It affects children of all ethnic groups and has an estimated incidence of 1 in 10,000 to 15,000 females (Kerr *et al.*, 1985, Hagberg *et al.*, 1993, Leonard *et al.*, 1997).

1.1.1 Identification of a new syndrome - RTT

Andreas Rett initially described RTT in 1966 but it was largely ignored until 1983, when Hagberg *et al.* published, the first description of a series of 35 patients, in English (Rett *et al.*, 1966, Hagberg *et al.*, 1983).

RTT is characterized by the cognitive regression (relating to conscious intellectual activity such as thinking, reasoning), deceleration of head growth, loss of purposeful hand use with the development of stereotypic hand movements, tremors (shaking from physical weakness), gait apraxia (loss of purposeful use of limb), and seizures (a sudden attack, as of disease) occurring after a period of normal development (Schanen *et al.*, 1999) .

There are necessary and supportive criteria to diagnose RTT (Table 1). Normal prenatal and perinatal period with normal developmental progress for the first 6-18 months of life is essential for the diagnosis. After normal head circumference at birth, there is deceleration that leads to microcephaly. Between 6 months to 3 years there is reduction or loss of acquired skills particularly purposeful hand use, vocalisation (producing sound with the voice) and communication skills.

The distinctive characteristic of RTT is the stereotypical hand movements such as hand wringing, hand washing, clapping, patting or other more bizarre hand automatisms. Gait ataxia (an inability to coordinate voluntary muscular movements that is necessary to walk on foot) is also an important feature. Supportive diagnostic criteria include breathing dysfunction, electroencephalographic abnormalities, spasticity, peripheral vasomotor disturbance, scoliosis (curvature of the spine), and growth retardation. If there is evidence of prenatal onset growth retardation, microcephaly at birth, an identifiable metabolic, degenerative or storage disorder, an acquired neurological disorder, retinopathy or optic atrophy, the clinical diagnosis of RTT is excluded. The nature of the condition is that of an evolving clinical phenotype, making the clinical diagnosis uncomplete until 2 to 5 years of age (Christodoulou *et al.*, 2001). An RTT patient displaying one of the features of the disorder is shown in figure 1.

Table 1 Rett Syndrome Diagnostic Criteria (Christodoulou *et al.*, 2001)

Necessary criteria	***Supportive criteria***	***Exclusion criteria***
1. Apparently normal prenatal and perinatal period 2. Developmental progress within normal range for the first 5- 6 months 3. Normal head circumference at birth, with subsequent deceleration 4. Reduction or loss of acquired skills (onset 6 months to 3 years) in particular purposeful hand use, vocalisation or speech (words) 5. Appearance of marked delay in development 6. Acquisition of hand stereotypes 7. Gait and or truncal apraxia(loss of purposeful use of hand) (by 4 years)	1. Breathing dysfunction 2. Periodic apnoea during wakefulness 3. Intermittent hyperventilation 4. Breath holding 5. Forced expulsion of air or saliva 6. EEG abnormalities 7. Slow wave background with intermittent rhythmical activity (3-5 Hz) 8. Epileptiform discharges, with or without clinical seizures 9. Spasticity, later with muscle wasting dystonia 10. Peripheral vasomotor disturbance 11. Scoliosis 12. Growth retardation 13. Hypotrophic, small, cold feet	1. Evidence of prenatal onset of growth retardation or microcephaly 2. Organomegaly or other evidence of storage disorder 3. Retinopathy or optic atrophy 4. Existence of identifiable metabolic or other neurodegenerative disorder 5. Acquired neurological disorder resulting from severe infection of head trauma

Figure 1. A girl with classical RTT phenotype.
Constant hand wringing, which is a typical symptom of RTT, is observed in this girl. (Patient 00-173's photo was taken with permission from the family)

1.1.1.1.Natural History

The natural history of a typical patient with classical RTT is characterized by the progression of four stages (see figure 1) (Hagberg *et al.*, 1986). The patients are normal at birth and exhibit normal development between 6-18 months. During **stage I**, **early onset of stagnation**, obtaining of new skills slows and patients frequently show autistic traits. Head growth slows and hypotonia is seen. After several months, **stage 2**, **the rapid regression stage** slowly develops. Previously acquired skills such as purposeful use of the hands and receptive and expressive language are lost and there is gait apraxia.

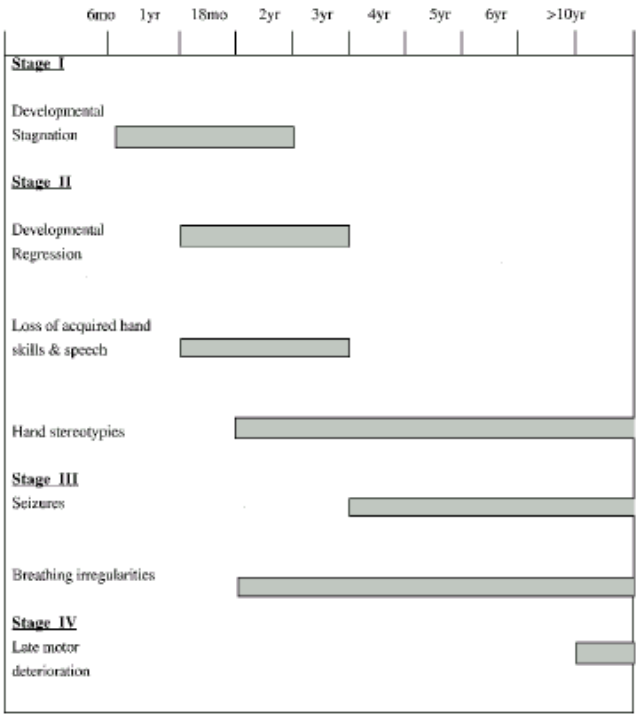


Figure 2. Age of onset of signs and symptoms in RTT (Christodoulou and Ellaway *et al.*, 2001)

Also several unusual behaviours begin. In **Stage 3**, **the pseudostationary stage**, child's ability about interacting with her environment increases as autistic features diminish. Patients develop an irregular respiratory pattern while awake. Seizures are most frequent during this stage and hand movements intensify. Somatic growth is poor and many patients develop osteopenia. **Stage 4**, **the late motor deterioration stage**, usually presents by 10 years of age and is characterized by reduced mobility. However there is no decline in cognition, communication or hand skills. Repetitive hand movements may decrease. Scoliosis is a prominent feature. Rigidity (stiffness) and dystonia (increased muscle tone with abnormal extremity) are characteristic. The majority of RTT patients survive into adulthood.

1.1.1.2.RTT Variants

Females who carry out all of the diagnostic criteria for RTT are classified as having typical or classical RTT. However, the clinical expression of RTT also includes atypical forms that may be more severe or mild in phenotype. Five possible RTT variants were described:

1. *Infantile seizure onset type: The* characteristic features of this form are predominance of seizures and onset of the disorder before 6 months (Hanefeld *et al.*, 1985).

2. *Congenital form:* This form appears early without a period of normal development, and involves congenital hypotonia and infantile spasms (Nomura *et al.*, 1985, Rolando *et al.*, 1985).

3. *Forme Fruste:* This is the milder form that experiences less severe regression and milder mental retardation and does not have seizures (Hagberg *et al.*, 1989).

4. *Late childhood regression:* In this case, regression has developed later and more gradually than classic RTT (Gillberg *et al.*, 1989).

5. *Preserved speech variant (PSV):* PSV shares with classic RTT some symptoms like stereotypical hand-washing activities but differs in that patients typically recover some degree of speech and hand use and usually do not show growth failure (De Bona *et al.*, 2000).

1.1.2. Hypothesis on the inheritance of the RTT gene

The genetic basis of Rett syndrome had been discussed extensively in the literature following the identification of the syndrome. Since 99.5% of all cases are sporadic, it was not easy to define the inheritance pattern (Hagberg *et al.*, 1983, Martinho *et al.*, 1990, Migeon *et al.*, 1995, Comings *et al.*, 1986, Ellison *et al.*, 1992, Zoghbi *et al.*, 1988). There were some clues about the possible genetic origin of the syndrome such as almost exclusive occurrence in females, high concordance rate among monozygotic twins while disconcordance among dizygotic twins, and presence of rare familial cases (Comings *et al.*, 1986, Ellison *et al.*, 1992, Zoghbi *et al.*, 1988, Engerstrom *et al.*, 1992, Schanen *et al.*, 1997, Sirianni *et al.*, 1998, Zoghbi *et al.*, 1990). The first hypothesis by the help of rare familial cases, which indicated inheritance through maternal lines and nonrandom patterns of X chromosome inactivation (XCI) in obligate carrier females, suggested that Rett syndrome is an X-linked dominant disorder caused by mutations in a gene that undergoes X inactivation (Schanen *et al.*, 1997, Sirianni *et al.*, 1998, Zoghbi *et al.*, 1990). There were some argumentative hypotheses against this first one. This inheritance pattern hypotheses included digenic inheritance of X-linked and autosomal loci (Buhler *et al.*, 1990) trinucleotide repeat expansions (Hofferbert *et al.*, 1997), mitochondrial inheritance (Ruch *et al.*, 1989, Dotti *et al.*, 1993, Lappalainen *et al.*, 1994, Haas *et al.*, 1995a, Haas *et al.*, 1995b, Tang *et al.*, 1997), and autosomal dominant inheritance with sex-limited expression (Killian *et al.*, 1986). Careful assessment of the familial cases shown in figure 3, and analysis of the X inactivation patterns in the putative carrier mothers, X-linked dominant inheritance became favourable again (figure 3). In addition, X inactivation studies performed in unaffected and obligate carrier females displayed skewed X inactivation which favours the mutant X, and

thus improves the phenotype (Willard *et al.*, 1996, Puck *et al.*, 1998). II-2 in Rett syndrome kindred 2, I-2 in Rett syndrome kindred 3, and I-2 in Rett syndrome kindred 4 are the examples of the obligate carriers of Rett syndrome who were mildly affected because of the skewed X chromosome inactivation (Migeon *et al.*, 1995, Schanen *et al.*, 1997, Sirianni *et al.*, 1998, Zoghbi *et al.*, 1990). Also in discordant monozygotic twins, the preferential inactivation of paternal X chromosome was reported (Migeon *et al.*, 1995)

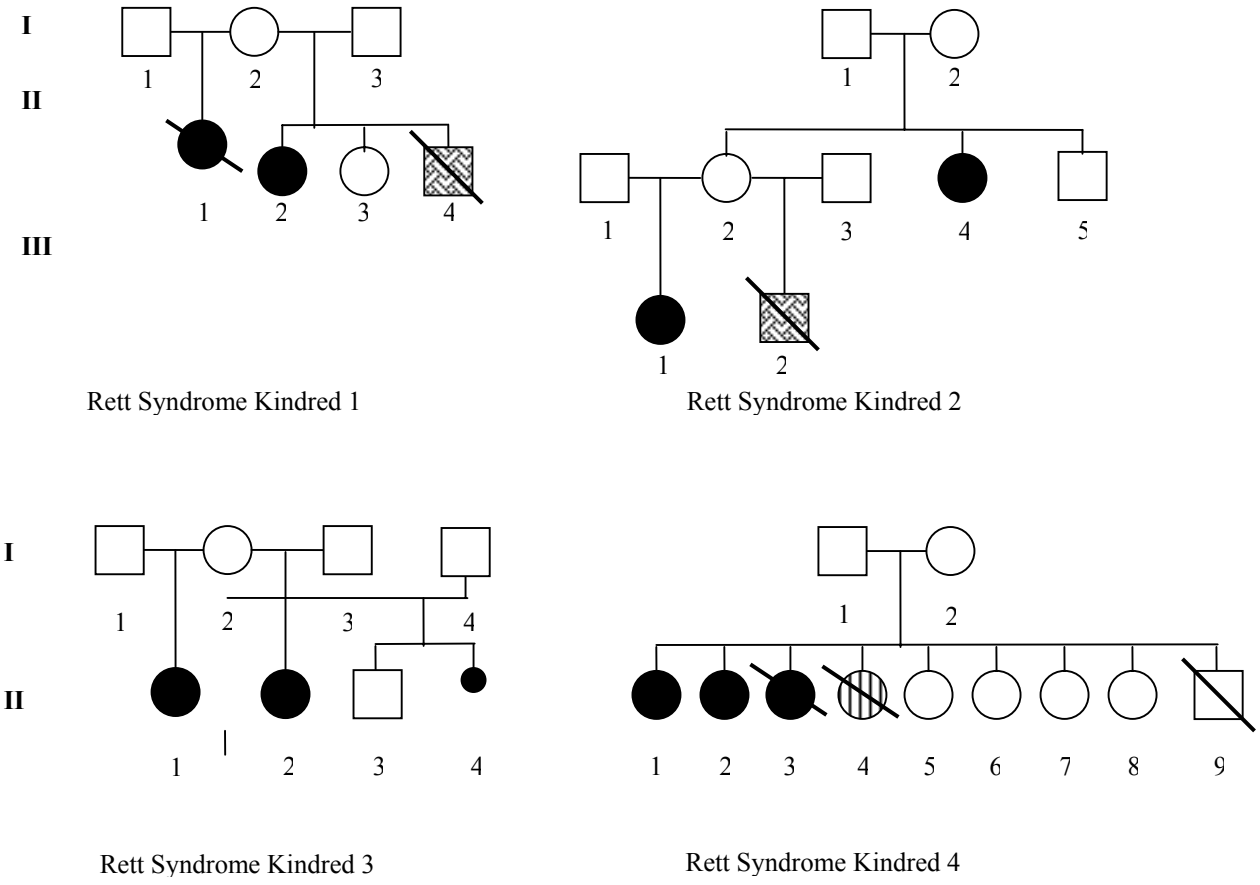


Figure 3. Pedigree for four RTT kindreds, which were used for mapping the locus.

Filled symbols: Probands with classic RTT phenotype; Hatched symbols: Male RTT patients (Schanen *et al.*, 1999).

1.1.3. Localisation of the RTT locus via exclusion mapping

In order to map a locus, 3 strategies could be performed. The first one is linkage analysis. This analysis was not proper for mapping RTT locus because 99.5% of all cases were sporadic so there were not enough families to perform linkage analysis. The second is cytogenetic analysis. It was promising, and conducted. But, classical banding techniques, FISH and Southern blotting did not detect any abnormalities such as translocations, microdeletions or duplications, which would be consistent with an X-linked dominant disorder (Fan *et al.*, 1999). The third one, exclusion mapping, was the most suitable method for the localisation of the RTT gene.

The basis of exclusion mapping is that because the related probands inherited the same mutation, the defective gene must lie in a region of the X chromosome that is shared by the probands (Schanen *et al.*, 1999). The first progress in mapping came from the studies of RTT kindreds 1, 2 and 3 (figure 3). These were maternally related half-sisters and aunt-niece pair in these families (Ellison *et al.*, 1992, Anvret *et al.*, 1990, Archidiacono *et al.*, 1991). Polymorphic X-linked markers were typed for RTT kindred 1, 2 and 3, and the region near the marker was excluded if the probands inherited different alleles. Subsequently, a Brazilian family (RTT kindred 4) was identified, which further narrowed the critical region (Figure 3 and 4). In general, using the affected sister pairs for exclusion mapping was risky because the mutation on the putative RTT gene could be transmitted through the paternal lineage if the father was a germline mosaic. But for RTT kindred 4, it was thought that the inheritance was maternal since there were affected and unaffected sisters and skewed X inactivation in the mother. With the help of these four RTT kindreds, the RTT

locus was mapped to Xq28, a particularly gene rich part of the genome, via exclusion mapping (Schanen *et al.*, 1997, Sirianni *et al.*, 1998, Schanen *et al.*, 1998).

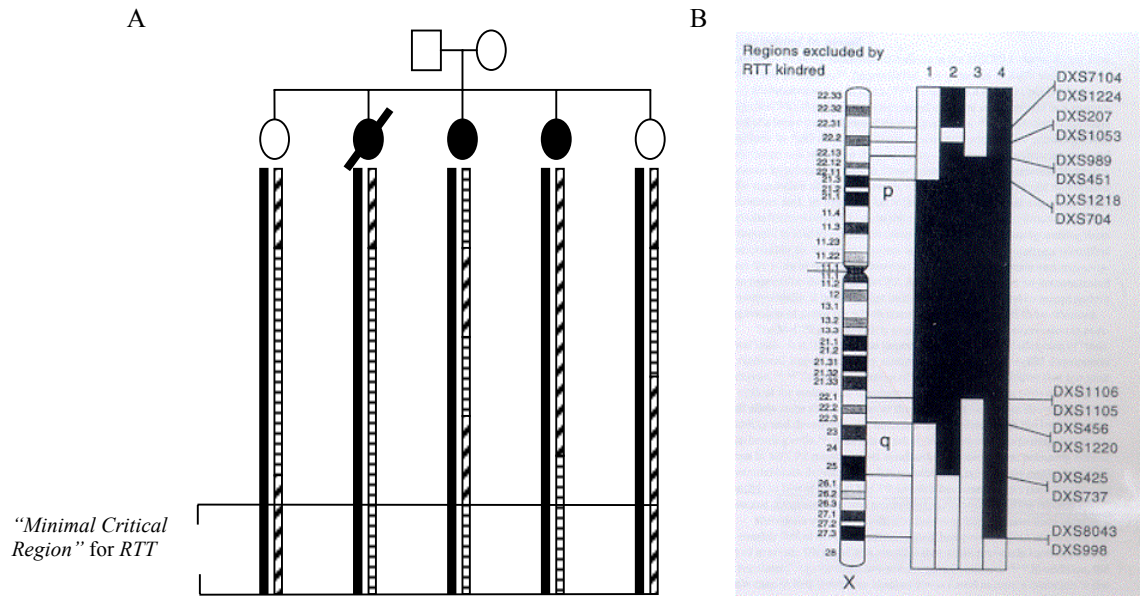


Figure 4. The exclusion mapping

A. Pedigree of the Brazilian family and schematic diagram of extended haplotype of each X chromosome. The only region of X chromosome concordant for RTT is indicated (Sirianni *et al.*, 1998).
B. The regions excluded by the four RTT kindreds is summarized. Filled bars indicate excluded regions. The loci flanking the discordant and concordant regions are shown on the right. Ideogram shows the approximate cytogenetic location (Schanen *et al.*, 1999).

1.1.4. Candidate gene screening

In many disorders, after genetic mapping, transcripts within critical region were identified and then were screened for mutations in probands. The Human Genome Project was instrumental in the increase of the number of genes that fall into the minimal critical region which would be used for mutation screening in relatively few families (Schanen *et al.*, 1999, Dragich *et al.*, 2000). In order to choose the candidate genes, the RTT pathology was used as a clue. In RTT, obvious pathology

was limited to the central nervous system (CNS) where there was evidence for abnormal development or maintenance of affected neurons. As a note, mosaicism for the mutation was not apparent at the cellular level (Armstrong *et al.*, 1998). Initially, attention was largely directed toward genes expressed predominantly in the CNS, and genes for neurotransmitter and their receptors, neural specific proteins were excluded (Heidary *et al.*, 1998, Percy *et al.*, 1998, Van Den Veyver *et al.*, 1998, Narayanan *et al.*, 1998, Cummings *et al.*, 1998, Wan *et al.*, 1998). However careful examination of female patients and more severely affected male patients indicated that the RTT gene was also important for the function of other tissues (Motil *et al.*, 1994; Haas *et al.*, 1997; Motil *et al.*, 1998). Finally, 14 years of search, Amir *et al.* and Wan *et al.* broke the silence in RTT in late summer 1999, and identified *MECP2* mutations in RTT probands both in familial and sporadic cases.

1.2. Methyl-CpG binding protein 2 (*MECP2*) gene

1.2.1. Identification of *MECP2* gene

CpG dinucleotides are nonrandomly formed at much of the heterochromatic regions of the chromosomes and the promoter regions of many genes. 60-90% of CpG nucleotides in mammalian genome are modified by methylation at the carbon 5 position. The remaining non-methylated CpGs are found in CpG islands that usually include functional promoters. Methylation of cytosine residues in CpGs is important both in stable silencing of heterochromatin and reversible regulation of gene expression, however, it is not important for the proliferation and *in vitro* differentiation of embryonic stem (ES) cells (Ng *et al.*, 1999a, Li *et al.*, 1992, Tate *et al.*, 1996).

Two models were suggested for transcriptional repression based on CpG methylation. According to the first model, DNA is bound by proteins, which preferentially interact with methylated CpG sites, and prevent binding of activators or basal transcription factors. Second model suggests that due to preferential interaction between methylated DNA, ubiquitous components of chromatin may alter chromatin structure and lead to transcriptional repression (Mostoslavsky *et al.*, 1997).

Initial efforts to identify protein-mediated CpG methylation-dependent repression led to the identification of *MECP1* (Meehan *et al.*, 1989, Boyes *et al.*, 1991). After a year, a second member of MECP family was identified and cloned in rat (Lewis *et al.*, 1992), and in the human (Adler *et al.*, 1995). This member was named as *MECP2*. Both of the members bind symmetrically methylated CpGs with a sequence-independent manner (Boyes *et al.*, 1991). Although *MECP1* required more than 10 CpG pairs to bind DNA (Boyes *et al.*, 1992), *MECP2* can bind singly methylated CpG pairs (Lewis *et al.*, 1992). Although there is functional homology between *MECP1* and *MECP2*, different binding specificity and expression patterns suggest that *MECP1* does not compensate for the loss of *MECP2* function (Dragich *et al.*, 2000).

Mecp2 gene was found to be X-linked in the mouse (Quaderi *et al.*, 1994), and later studies placed *MECP2* gene to human X chromosome in Xq28 between the interleukin I receptor-associated kinase (*IRAK*) and the color vision (*RCP*/*GCP*) loci. The *MECP2* gene spans 76 kb and is composed of four exons that is transcribed from telomere to centromere with 1461 nucleotide coding sequence

(figure 5). The 5' non-coding exon was identified recently by Reichwald *et al.*2000, *MECP2* is subjected to X-inactivation (D'esposito *et al.*, 1996).

Figure 5. *MECP2* is flanked by *IRAK* and *RCP* loci.

The direction of the arrows indicates the orientation of the transcription (Dragich *et al.*, 2000)

Although, expression of *MECP2* was at low levels early in development, it was expressed ubiquitously in embryonic and adult tissues (Meehan *et al.*, 1992). *MECP2* has three transcripts, which vary in length. Their lengths are respectively 1.8kb, ~7.5kb and ~10 kb. By the different use of polyadenylation signals in 3'UTR, these three transcripts are formed. Although there is tissue-specific variation in expression, short (1.8 kb) and long (10 kb) transcripts are present in most of the tissues. The long transcript is found at higher levels than the shorter one in brain and spinal cord. They are expressed similarly in kidney, thyroid, lung, gastrointestinal tract and adrenal glands. The short one is expressed at higher levels in skeletal and cardiac muscle, lymphoid tissues, liver and placenta (D'esposito *et al.*, 1996, Reichwald *et al.*, 2000). Although, there is a low level of expression of the long transcript in the developing nervous system, the expression is increased in postnatal hippocampus and olfactory bulb (Coy *et al.*, 1999). Because of the identical half-lifes of the short and the long transcripts, the difference between the functions of these transcripts is not fully understood yet (Reichwald *et al.*, 2000).

1.2.2. The structure and function of MeCP2

MeCP2 is an abundantly expressed nuclear protein which is associated with 5-methyl-rich heterochromatin (Tate *et al.*, 1996, Nan *et al.*, 1997). Its 486 amino acids consist of four functional domains: (1) a **methyl-CpG binding domain** (MBD; 85 amino acids in length) which is necessary to bind 5-methyl cytosine in the major groove of DNA in the presence or absence of assembled chromatin (Nan *et al.*, 1993, Wakefield *et al.*, 1999); (2) a **transcriptional repression domain** (TRD; 104 amino acids in length) which interacts with corepressor Sin3A to recruit histone deacetylases 1 and 2 (HDAC1and 2)(figure 6) (Nan *et al.*, 1998b, Jones *et al.*, 1998); (3) a **nuclear localization signal** (NLS) which may be responsible for the transport of MeCP2 into the nucleus (Nan *et al.*, 1996a, Nan *et al.*, 1996b) and (4) a **C-terminal segment** which facilitates its binding to the nucleosome core (Chandler *et al.*, 1999).

MeCP2 represses transcription through a mechanism that involves binding to CpGs and recruitment of HDACs to modify chromatin structure. This leads to deacetylation of histones which allows DNA to wind more tightly around the histone, and prevents the access of the transcription machinery to the promoters (Jones *et al.*, 1999, Wolffe *et al.*, 2000). However MeCP2 does not always require deacetylase activity to repress transcription (Nan *et al.*, 1998a, Kaludov *et al.*, 2000, Yu *et al.*, 2000). There is a interaction between MeCP2 and the transcriptional machinery, which comprises TFIIB and E2F (Di Fiore *et al.*, 1999). This interaction is an evidence for MeCP2 to repress transcription at a distance (>500 bp) (Nan *et al.*, 1997, Nan *et al.*, 1998a, Kaludov *et al.*, 2000). Since, MeCP2 binds to the matrix attachment sites, it may have a role in the architecture of silenced chromatin (Buhrmester *et al.*, 1995, Andrulis *et al.*, 1998). These findings indicate that

MeCP2's role in nucleus is complex and mediate transcription through overlapping mechanisms.

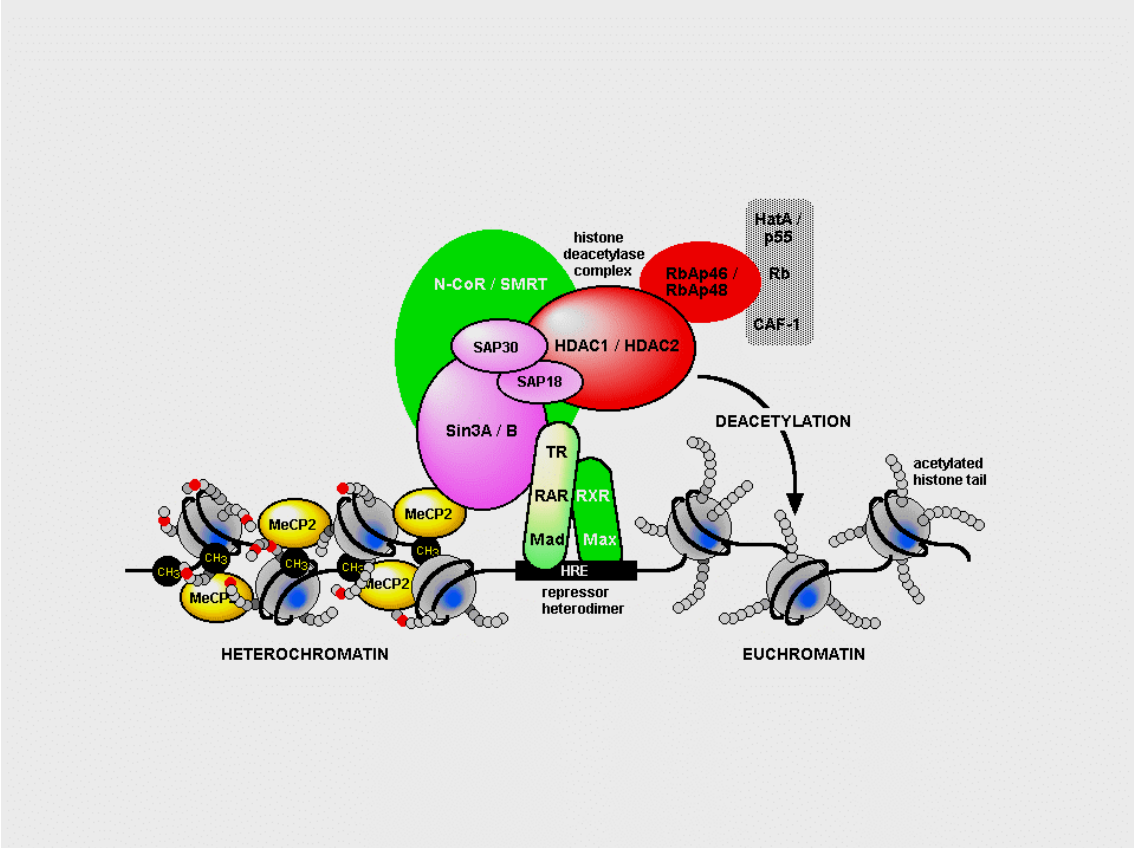


Figure 6. Schematic representation of the interaction between MeCP2 and histone deacetylase complex.

1.2.3. Mutations of *MECP2*

The mutation analyses have been focused on the coding region till now because the untranslated regions of the gene is too long (particularly intron 1, 2 and 3'UTR). *MECP2* mutations have been reported in more than 300 RTT patients, and up to %80 of the sporadic and %50 of the familial cases were found to have mutations. As a consequence of the sporadic occurrence of RTT, most mutations are de novo. However, there are eight recurrent mutations namely R106W, R133C, T158M, R168X, R255X, R270X, R294X, and R306C resulting from C to T transitions that account for ~65% of mutations in patients (Amir *et al.*, 1999, Wan *et al.*, 1999, Huppke *et al.*, 2000, Cheadle *et al.*, 2000, Bienvenu *et al.*, 2000, Obata *et al.*, 2000, Hampson *et al.*, 2000, Buyse *et al.*, 2000, Inui *et al.*, 2001, Vacca *et al.*, 2001, Bourdon *et al.*, 2001, Nielsen *et al.*, 2001, Erlandson *et al.*, 2001).

The deletions found in the 3' end of exon 4 which account for 10% of the known mutations are important for the function presumably because they are involved in interaction of *MECP2* with nucleosome core (Chandler *et al.*, 1999). More than 64 distinct mutations have been identified; most of them are nonsense (approximately 41) although missense mutations (approximately 23) affecting highly conserved amino acid residues are also seen. Figure 7 shows the majority of the mutations found in *MECP2* gene and the mutations already associated with RTT is summarized in appendix 3.

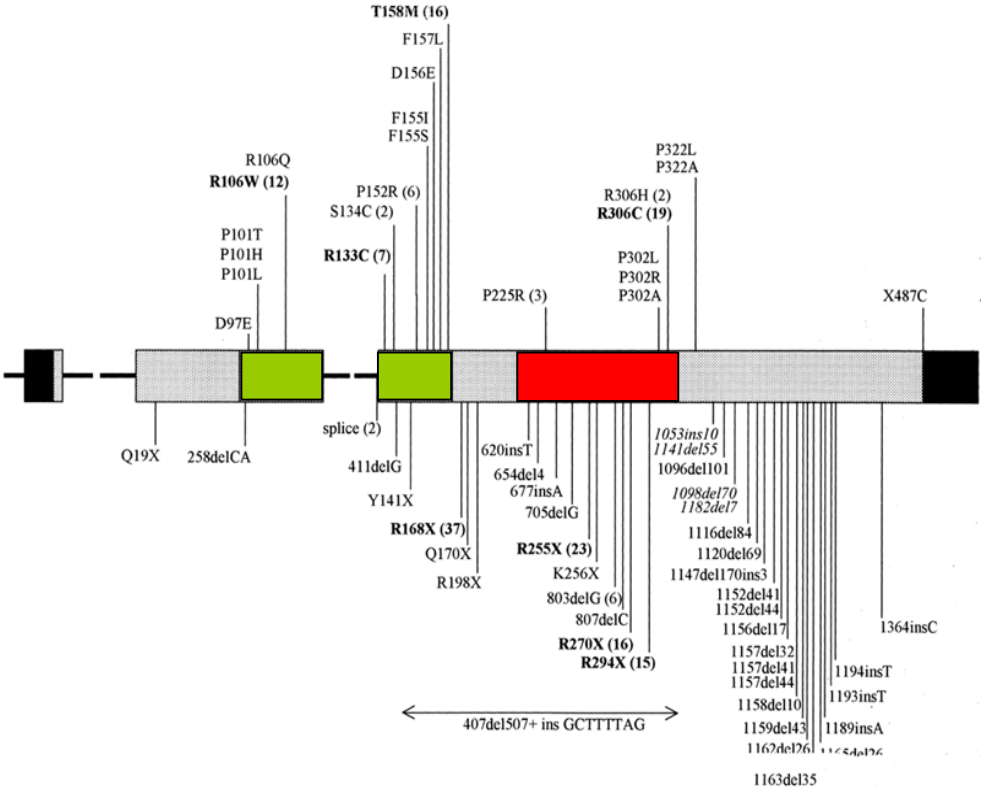


Figure 7. Mutations identified in *MECP2*.

Exons 2-4 and the mutations of *MECP2* is depicted in the diagram. Noncoding regions is in black. MBD is in green and TRD is in red. Missense mutations are listed above and nonsense mutations below the exons. The number of occurrence is written in parenthesis near the recurrent mutations (Amir *et al.*, 2000).

There is a high proportion of C to T transitions at CpG sites because CpG dinucleotides are hypermutable, and germline and somatic mutations are common at these sites (Rideout *et al.*, 1990). The mechanism that causes this transition may involve methylation of 5' cytosine via methyltransferase and spontaneous deamination of 5-methylcytosine to thymine. C to T or G to A (on antisense) transitions constitute ~55% single-nucleotide substitutions (Krawczak *et al.*, 1996). There is a high level of CpG methylation in male germ cells. Also the whole X chromosome, including *MECP2*, is methylated (Girard *et al.*, 2001). The

coding sequence of *MECP2* contains 35 CpGs with a C to T transition possibility. Exon 1 does not contain CpG pairs, which in part explains why no mutation has been found in this exon.

1.2.4. Polymorphisms of *MECP2*

By using familial cases, ten different polymorphisms have been identified (Amir *et al.*, 1999, Wan *et al.*, 1999, Cheadle *et al.*, 2000, Buyse *et al.*, 2000, Amir *et al.*, 2000a, Orrico *et al.*, 2000).

Table 2 *MECP2* polymorphisms identified.

Variant and Exon	Nucleotide Change	Amino Acid Change	Domain
3	375 C to A	I125	MBD
4	582 C to T	S194	
4	608 C to T	T203M	
4	843 C to T	A281	TRD
4	897 C to T	T299	TRD
4	984 C to T	L328	
4	1189 G to A	E397K	
4	1197 C to T	P399	
4	1233 C to T	S411	
4	1330 G to A	A444T	

1.2.5. The effect of the mutations on the function of *MECP2*

Normal function of MeCP2 includes binding to 5mCs in the promoters, recruiting deacetylase complex, and repressing transcription. When there is a mutation, MeCP2 no longer can bind to 5mC and can not recruit deacetylase complex. So, transcriptional noise from downstream genes appears to be likely (Van Den Veyver *et al.*, 2000) (figure 8).

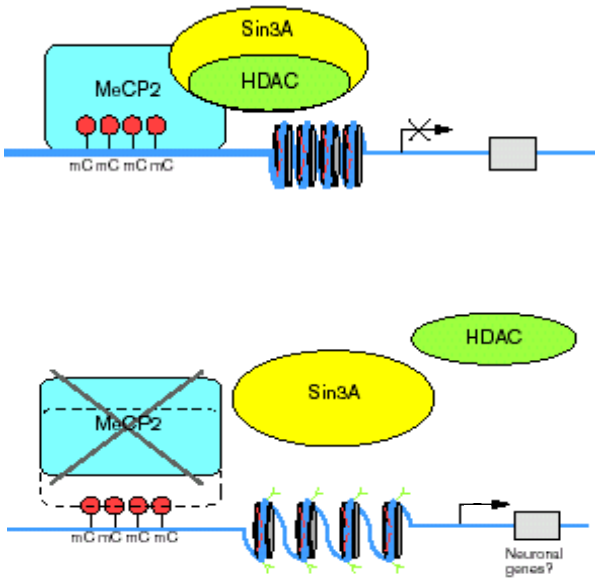


Figure 8. Schematic representation of normal function of MeCP2 and the effects of mutations to MeCP2 function (Van Den Veyver *et al.*, 2000)**.**

MECP2 is expressed during organogenesis throughout the embryo, and, later most strongly in the hippocampus (Coy *et al.*, 1999). Therefore, mutations of this gene could impair several organs. However, the *MECP2* related genes that have transcriptional repression activity and are members of repressor complexes could compensate for MeCP2 dysfunction in some tissues other than the brain. These genes, *MBD1, MBD2, MBD3*, and *MBD4* have been mapped to autosomes; and have similar MBDs, but not TRD domains (Hendrich *et al.*, 1999; Bird *et al.*, 1999; Ng *et al.*, 1999; Wade *et al.*, 1999) (figure 9).

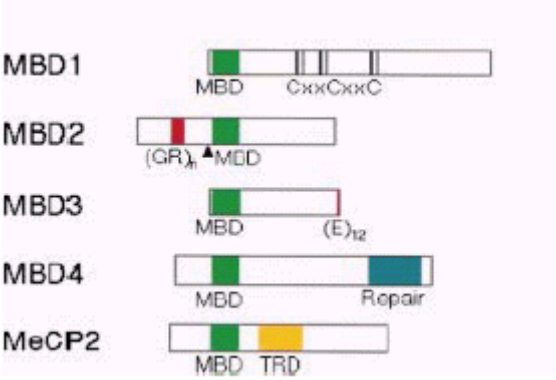


Figure 9. MBD protein family

The MBD protein family shares conserved methyl-CpG binding domains. CxxCxxC domain is found in MBD1. $(GR)_n$ represents, glycine-arginine repeats, and (E) represents glutamic acid repeats. The "repair" domain of MBD 4 is a TG mismatch glycosylase. TRD (transcriptional repression domain) is found only in MeCP2 (Bird *et al.*, 1999).

In general, missense mutations are localized within the MBD domain, except for R306C, which is found within the TRD, and impair selectivity for methylated DNA. Nonsense mutations, which truncate all or some part of the TRD domain, affect the ability to repress transcription, and lead to a decreased level of stability (Yusufzai *et al.*, 2000; Ballestar *et al.*, 2000). Deletions are found within the C-terminus, and affect the stability of the protein.

Majority of the nonsense mutation in the 5' end of the coding sequence of *MECP2* are proposed to result in the degradation of the mRNA molecule by a mechanism called nonsense mediated decay (NMD). This process monitors the mRNAs for errors during gene expression and degrades them (Leeds *et al.*, 1992 a,b). In contrast, similar mutations within the last exon may by-pass this pathway and result in the production of a truncated protein (Zhang J., 1998). Since majority of the mutations are nonsense and lie within the last exon, they are expected to escape the NMD pathway. In conjunction with this observation, a decrease in disease severity was noted in cases that had truncating mutations within or downstream of TRD when compared with the mutations within the N-terminal region. The nonsense mutations L138X, R168X, E235X, R255X, R270X, V288X, and R294X were found to lead to the truncation of the TRD, and affect the ability to repress transcription (Yusufzai *et al.*, 2000). A mutation resulting in the most truncated protein was reported from an autistic RTT patient with a relatively mild phenotype. This Q19X mutation led to a gene product of only 19 amino acids. This example indicates that the premature truncation at the beginning of the protein does not have to be the reason for a severe phenotype (Kim *et al.*, 2000; Nielsen *et al.*, 2001).

The three recurrent RTT missense mutations, R106W, R133C, and F155S have a greatly reduced (> 100-fold) affinity to the methylated DNA, which is consistent with the impairment selectivity for binding to methylated DNA (Yusufzai *et al.*, 2000; Ballestar *et al.*, 2000). Another recurrent missense mutation, T158M, which substitutes thr with met on the loop structure outside the DNA-binding domain, shows only a small reduction (2-fold) in affinity to methylated DNA. However, T158 in *MECP2* is conserved from *Xenopus* to human and not present in the other MBD family members, which suggest that this residue has a precise role not related with its methyl-CpG binding activity (Dragich *et al.*, 2000).

C-terminal region of *MECP2* is required for protein stability (Yusufzai *et al.*, 2000). Interestingly, this region shows an overall homology of 35% identity, and 50% positivity in a 75 amino acid region with two brain-specific factors, brain-specific factor-1 (BF-1) and fork head 4 (FKH4), which are members of the fork head family. Their role is restricted to developing telencephalon. This subregion overlaps with -COOH terminus of *MECP2*, which has been shown to facilitate MeCP2 binding to DNA (Chandler *et al.*, 1999).

1.3. *MECP2* and X-linked mental retardation

Mental retardation is related with substantial limitations in mental functioning. In OMIM (Online Mendelian Inheritance in Man Database), 937 entries contained the term "MR", corresponding to 220 autosomal dominant, 437 autosomal recessive, 159 X-linked, and 121 non classified conditions. IQ is used for measuring MR; 50<IQ<70 means mild MR, and IQ<50 means severe MR. Approximately 2-3% of the population has an IQ below 70. MR can be subdivided into two: syndromic, which is characterized with consistent and distinctive clinical finding (fragile-X is the most common one); and non specific (MRX) if MR is the only primary symptom among affected individuals. X-linked non specific MR represents 5% of all MRs. Eight genes have been identified which cause X-linked MR when mutated. These genes are *OPHN1, GDI1, PAK3, ILRAPL, TM4SF2, VCX-A* and *ARHGEF6* (figure 10). However, their incidence is very low, being around 0.5-1% of MRX (Toniolo *et al.*, 2000; Castellvi-Bel *et al.*, 2001).

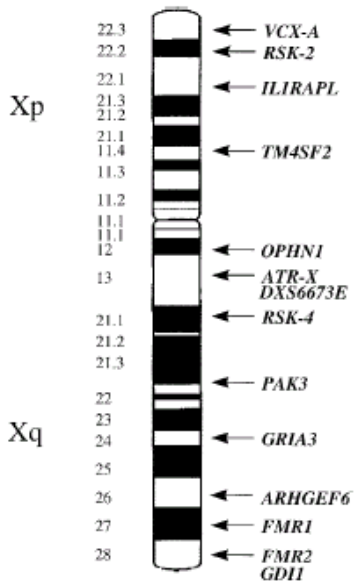


Figure 10. The MR genes that are localized on the X chromosome (*MECP2* not included) (Castellvi *et al.*, 2001).

Recently, mutations in *MECP2* have been identified in four non specific X-linked mental retardation families with multiple affected individuals. In the first family A140V mutation was identified in all of the four severely affected males and two mildly affected females (Orrico *et al.*, 2000). E406X mutation was identified in a three generation family in which two affected males display severe MR and progressive spasticity (Meloni *et al.*, 2000). E137G mutation was found in another MR family, and finally R167W mutation was found in a three-generation family with four non-specific mentally retarded males (Couvert *et al.*, 2001). So far, these mutations have not been reported in any one of the typical RTT cases.

There is a high frequency of mutations in *MECP2* (~2%) when compared with the other non specific X-linked MR genes (%0.5-1%). This finding indicates that *MECP2* gene mutations are important for the MR phenotype (Couvert *et al.*, 2001).

In addition to MR, *MECP2* mutations have been identified in patients with Angelman syndrome phenotype (Imessaoudene *et al.*, 2001; Watson *et al.*, 2001).

1.4. Male cases

Although RTT exclusively affects females, rare male cases have also been reported. Until now, five reports described mutations in male cases (Wan *et al.*, 1999, Clayton-Smith *et al.*, 2000, Meloni *et al.*, 2000, Orrico *et al.* 2000, Villard *et al.*, 2000). Four of them are rare familial cases. First one described a male child with congenital encephalopathy who survived to age >1 year old (Wan *et al.*, 1999). Second described four brothers with severe nonspecific MR and movement disorders

(Orrico *et al.*, 2000). Third described two men affected by severe mental retardation and progressive spasticity (Meloni *et al.*, 2000). Fourth described two brother who died because of severe neonatal encephalopathy (Villard *et al.*, 2000), and the fifth described a sporadic case in which a boy affected by a nonfatal neurodevelopmental disorder who has somatic mosaicism for a *MECP2* mutation (Clayton-Smith *et al.*, 2000).

However *MECP2* mutations are considered to be lethal for males. In addition, sex-limited expression of RTT can be explained by the occurrence of the *de novo* X-linked mutations exclusively in the male germ cells which results in affected daughters. With this hypothesis, the absence of affected males can be explained by the fact that sons do not inherit their X-chromosome from their fathers. The frequency of male-germ-line transmission of the mutation was found as 71% (Girard *et al.*, 2001) and 96.3% (Trappe *et al.*, 2001). These findings suggest that male patients are naturally protected from *de novo MECP2* mutations.

1.5. Mouse models for RTT

The unresolved issue in the pathogenesis of RTT is whether the disease is the result of a dysfunction of postnatal neurons when the symptoms become apparent or a prenatal developmental abnormality with postnatal phenotypic appearance of the disease (Chen *et al.*, 2001). The most appropriate approach to address this issue appears to be mouse studies.

The first mouse study indicated that *Mecp2* is essential for development, and its disruption leads to embryonic lethality (Tate *et al.*, 1996). Recent studies oppose this finding and demonstrated that *Mecp2*-null mice are

viable, and show some of the symptoms of RTT at approximately six weeks of age (Chen *et al.*, 2001, Guy *et al.*, 2001).

In both of these studies conditional knock-out technology was used to delete exon 3 or exons 3 and 4 of *MECP2*. Both in *Mecp2*-null mice and mice in which *Mecp2* was deleted in brain showed some of the symptoms of RTT such as tremor, heavy breathing, and cold extremities indicating autonomic abnormalities that are also characteristic for human RTT patients. The most consistent changes with the human RTT in mutant mice were smaller brain size and general reduction of neuronal cell size (Chen *et al.*, 2001, Guy *et al.*, 2001).

1.6. Epigenetic regulation of gene expression and RTT

Methylation of CpG dinucleotides is necessary for transcriptional repression and underlies the processes of X-inactivation, genomic imprinting, tissue-specific and developmental regulation of gene expression (Cross *et al.*, 1995).

1.6.1. X-inactivation

Dosage of X-linked genes is kept equal between females and males by random X inactivation in each cell early in embryogenesis in females (Lyon *et al.*, 1986). The exception for that is the genes in pseudoautosomal region which normally escapes from X-inactivation and have functional homologues on Y chromosome (Schneider-Gadicke *et al.*, 1989, Goodfellow *et al.*, 1984, Fisher *et al.*, 1990).

Two X-inactivation mechanisms, which can lead to RTT, were hypothesized. The first, a mechanism in which the abnormality in X-chromosome

inactivation leading to functional disomy of an X-linked gene(s), was proposed to explain exclusive occurrence of RTT in females (Riccardi *et al.*, 1986). The existence of male patients argues strongly against this disomy model (Schanen and Francke, 1998).

The second is a more likely model which suggests that X-inactivation patterns influence the phenotypic expression of RTT (Schanen *et al.*, 1999). Random X-inactivation pattern was found in most of the RTT cases (Zoghbi *et al.*, 1990, Anvret *et al.*, 1990, Nielsen *et al.*, 2001, Amir *et al.*, 2000a, Webb *et al.*, 1993, Camus *et al.*, 1996). Skewed X inactivation, which leads to differences in phenotypic expression was also observed in several non penetrant or mildly affected obligate carrier females, and in an unaffected twin. These results indicate that non random pattern of X inactivation protects against the consequences of *MECP2* mutations (Migeon *et al.*, 1995, Schanen *et al.*, 1997, Sirianni *et al.*, 1998, Zoghbi *et al.*, 1990, Wan *et al.*, 1999, Amir *et al.*, 2000a, Krepischi *et al.*, 1998, Villard *et al.*, 2001). Recently, a case of RTT with 46,X,r(X) in which complete skewed inactivation of the ring was shown. Interestingly, no mutations were found in the *MECP2* gene present in intact X. This finding suggested that, there could be two loci related with RTT, one with the *MECP2* locus which mutations predominantly cause sporadic RTT. The second is an unidentified locus in which mutations cause mildly affected or unaffected carriers or familial cases (Rosenberg *et al.*, 2001).

To evaluate the X-inactivation pattern, a rapid PCR methylation assay has been developed for androgen receptor gene locus, where methylation of AR locus correlates with X-inactivation. In AR assay, which is widely used, the paternal

allele can be distinguished from the maternal copy through the polymorphism of the trinucleotide repeats in the locus (Allen *et al.*, 1992)

1.6.2. Genomic Imprinting

Genomic imprinting is an epigenetic form of gene regulation that determines expression or repression of genes according to their parental origin (Reik *et al.*, 1998, Jirtle *et al.*, 1999). This mechanism results in monoallelic expression of the imprinted genes. More than 25 imprinted genes have been identified. These genes have a role in fetal and placental growth, cell proliferation, and adult behaviour (Jirtle *et al.*, 1999, Barlow *et al.*, 1995).

Methylation at CpG sites controls the multi step imprinting process. In this process, the chromosome is methylated during gametogenesis or in the zygote depending on its parental origin. There is continuity in methylated state during cell division and differentiation and the transcriptional machinery recognises the methylated CpGs that result in monoallelic expression (Pfeifer *et al.*, 2000). Since methylation-related transcriptional silencing underlies genomic imprinting, a role for MeCP2 in this process can be thought. However no data has been found about the over or underexpression of imprinted genes as a consequence of *MECP2* mutations (Wan *et al.*, 1999).

1.6.3. Developmental regulation of gene expression

During development, cells and tissues differentiate due to regulated gene expression. This regulation of gene expression can be performed in two levels (Minie *et al.*, 1992). First is at the level of individual interaction of trans-acting factors with local promoters and enhancers. The stage-specific transcription factors, which have a role in the regulation of B cell differentiation during development is a

good example of this process (Hagman *et al.*, 1994). The second is at the level of chromatin structure. The developmentally regulated changes in histones determines the repression of specific genes (Wolffe *et al.*, 1996).

Since MeCP2 is a global transcriptional repressor, the mutations of *MECP2* can disrupt gene regulation in development by affecting trans-acting factors.

1.6.4. Tissue-specific gene expression

Although, the DNA content of all eukaryotic cells is identical, there are different cell types. The thing that makes this difference is the pattern of the genes, which are expressed in the cell. Briefly, the genes that are expressed define the function of the cell. Common housekeeping genes, which are expressed in all cell types, perform the essential cell function. The expression of other genes is restricted to specific cell type with the help of tissue-specific gene expression process (Strachan *et al.*, 1996).

MeCP proteins bind to methylated CpG at a promoter and prevent expression of the gene. This process constitutes the cornerstone of the tissue specificity. It can be thought as the mutations of *MECP2* can disrupt the tissue specific gene expression. But this hypothesis is highly unlikely since with impaired tissue- specific gene expression process, the fetus would most probably be not viable. Also there are functional and structural homologues of MeCP2 protein, which can take over the impaired function of MeCP2.

1.7. Aim and Strategy

The Rett syndrome project in our laboratory has the main aim of studying the possible disturbances in DNA-methylation dependent gene silencing which may be a new disease mechanism in human. My specific aims in this project are (1) Conformation of RTT diagnosis by DNA analysis. For this purpose recurrent *MECP2* mutations will be analysed by restriction enzyme analysis and confirmed via automated sequencing. (2) Correlation of the phenotype (the symptoms) with the genotype (the mutations). (3) Correlation of the X chromosome inactivation patterns with the clinical severity.

2. Materials and Methods

2.1. Materials

2.1.1. Patient Samples

Collaborating physicians at Hacettepe University Medical Faculty (Ankara, Turkey) referred Rett Syndrome patients to Bilkent University, Faculty of Science, Molecular Biology and Genetics Department (Ankara, Turkey). Blood samples were collected in tubes containing EDTA. Informed consent was obtained from the parents of the patients.

2.1.2. Oligonucleotides

The primers used in the polymerase chain reactions (PCR), and the cycle sequencing reactions were synthesized on the Beckman Oligo 1000 M DNA synthesizer (Beckman Instruments Inc., Fullerton, CA, USA) at Bilkent University, Faculty of Science, Department of Molecular Biology and Genetics (Ankara, Turkey). The primer sequences used for the analysis of *MECP2* gene and *AR* gene are given in Table 3 and 4.

Table 3 Sequence of the *MECP2* primers

Exons	Name	Sequence (5'→3')	Expected Size (bp)	[$MgCl_2$] (mM)
RTT 3 F	GA 526	CCTGGTCTCAGTGTTCATTG	597	1.5
RTT 3 R	GA 527	CTGAGTGTATGATGGCCTGG		
RTT 4.1 F	GA 530	TTTGTCAGAGCGTTGTCACC	380	1.5
RTT 4.1 R	GA 531	CTTCCCAGGACTTTTCTCCA		
RTT 4.2 F	GA 532	AACCACCTAAGAAGCCAAA	380	1.5
RTT 4.2 R	GA 533	CTGCACAGATCGGATAGAAGAC		
RTT 4.3 F	GA 534	GGCAGGAAGCGAAAAGCTGAG	366	1
RTT 4.3 R	GA 535	TGAGTGGTGGTGATGGTGGTGG		
RTT 4.4 F	GA 536	TGGTGAAGCCCCTGCTGGT	414	1.5
RTT 4.4 R	GA 537	CTCCCTCCCCTCGGTGTTTG		
RTT 4.5 F	GA 538	GGAGAAGATGCCCAGAGGAG	386	1.5
RTT 4.5 R	GA 539	CGGTAAGAAAAACATCCCCAA		

Table 4 Sequence of the *AR* primer pair

Exons	Name	Sequence (5'→3')	Expected Size (bp)	[$MgCl_2$] (mM)
RS-6	GA542	GTCCAAGACCTACCGAGGAG	280	1
RS-7	GA543	CCAGGACCAGGTAGGCTGTG		

2.1.3. Chemicals and Reagents

The chemicals and reagents used in this project were purchased from the following sources:

Reagent	Supplier
Acrylamide	Sigma, St.Louis, MO, USA
Acetic Acid	Carlo Erba, Milano, Italy
Agarose	Basica LE, EU
Ammonium persulfate	Sigma, St.Louis, MO, USA
Bisacrylamide	Sigma, St.Louis, MO, USA
Boric acid	Sigma, St.Louis, MO, USA
Bromophenol blue	Sigma, St.Louis, MO, USA
Chelex	BioRad,Hercules, CA, USA
Chloroform	Carlo Erba, Milano, Italy
Ethanol	Merck, Frankfurt, Germany
Ethidium bromide	Sigma, St.Louis, MO, USA
Ficoll Type 400	Sigma, St.Louis, MO, USA
Formamide	Sigma, St.Louis, MO, USA
Glycerol	Carlo Erba, Milano, Italy
Hydrogen peroxide 40%	Carlo Erba, Milano, Italy
Isoamyl alcohol	Carlo Erba, Milano, Italy
Metaphor Agarose	FMC BioProd, Rockland, USA
NuSieve 3:1 Agarose	Basica LE, EU
Silver Nitrate	Sigma, St.Louis, MO, USA
Phenol	Carlo Erba, Milano, Italy
Proteinase K	Appligene-Oncor, USA

Reagent	Supplier
QIAquick PCR purification kit	Qiagen, Chatsworth, CA, USA
Sodium acetate	Carlo Erba, Milano, Italy
Sodium chloride	Sigma, St.Louis, MO, USA
Sodium dodecyl sulfate(SDS)	Sigma, St.Louis, MO, USA
Sodium hydroxide	Sigma, St.Louis, MO, USA
TEMED	Carlo Erba, Milano, Italy
TrisHCl	Sigma, St.Louis, MO, USA
Trisodium citrate	Sigma, St.Louis, MO, USA
Xylene cyanol	Sigma, St.Louis, MO, USA
pUC Mix Marker, 8	MBI Fermentas Inc., Amh, NY, USA,

2.1.4. Restriction Enzymes

The restriction enzymes used in this project with their recognition and restriction sites and the composition of the recommended buffers are listed in table 5. The enzymes were obtained from the designated suppliers and used according to the manufacturers' instructions.

Table 5 Restriction Enzymes Used for Mutation Detection

Restriction Enzyme	Recognition Site	Buffer (1X)	Supplier
NlaIII	5'-CATG↓-3' 3'-↑GTAC-5'	NE Buffer 4 50mM potassium acetate 20mM Tris acetate 10mM magnesium acetate 1mM DTT	Biolabs Beverly, MA, USA
NlaIV	5'-GGN↓NCC-3' 3'-CCN↑NGG-5'	NE Buffer 4 50mM potassium acetate 20mM Tris acetate 10mM magnesium acetate 1mM DTT	Biolabs Beverly, MA, USA
HphI	5'-GGTGA $(N)_8$↓-3' 3'-CCACT $(N)_7$↑-5'	Buffer B^+ 10mM Tris-HCl 10mM$MgCl_2$ 0.1mg/ml BSA	MBI Fermentas Inc. Amherst,NY,USA
HhaI	5'-G↓CGC-3' 3'-CGC↑G-5'	Buffer Y^+ 33mM Tris acetate 10mM magnesium acetate 66mM potassium acetate 0.1 mg/ml BSA	MBI Fermentas Inc. Amherst,NY,USA
Hinf I	5'-G↓ANTC-3' 3'-CTNA↑G-5'	Buffer Y^+ 33mM Tris acetate 10mM magnesium acetate 66mM potassium acetate 0.1 mg/ml BSA	MBI Fermentas Inc. Amherst,NY,USA
Eco130I (StyI)	AA 5'-C↓CTTGG-3' 3'-GGAAC↑C-5' TT	Buffer Y^+ 33mM Tris acetate 10mM magnesium acetate 66mM potassium acetate 0.1 mg/ml BSA	MBI Fermentas Inc. Amherst,NY,USA
HpaII	5'-C↓CGG-3' 3'-GGC↑C-5'	Buffer Y^+ 33mM Tris acetate 10mM magnesium acetate 66mM potassium acetate 0.1 mg/ml BSA	MBI Fermentas Inc. Amherst,NY,USA

2.1.5. Polymerase Chain Reaction materials

The kits, which were used in PCR reaction, were obtained from MBI Fermentas Inc. (Amherst, NY, USA). Kits contained *Thermus aquaticus* DNA polymerase (5U/µl), 10X PCR buffer (100 mM Tris-HCl (pH 8.8 at 25 °C), 500 mM KCl, 0.8% Nonidet P40), 25 mM $MgCl_2$ solution, and 10 mM dNTP mix (one ml of 10 mM dNTP solution contains 10µmol each of dATP, dCTP, dGTP, dTTP). PCR reactions were performed in 0.2 ml Thermowell^{TM} tubes (Corning Costar Corp., Cambridge, MA, England) using the Gene Amp PCR system 9600 (Perkin Elmer, Foster City, CA, USA).

2.1.6. DNA sequence analysis materials

Cycle sequencing reaction was performed using the ABI PRISM^{TM} Ready reaction Dye Terminator Cycle Sequencing Kit (ABI, Perkin Elmer, Foster City, CA, USA). The sequencing kit contained terminator premix with A-dye terminator, C-dye terminator, G-dye terminator, T-dye terminator; dITP, dATP, dCTP and dTTP; Tris-HCl (pH 9.0); $MgCl_2$; thermal stable pyrophosphatase; and AmpliTaq DNA polymerase, FS (8 U/µl). Each kit also contained a PGEM ® 3 Zf(+) control template (0.2 µg/µl) and -21 M13 forward primer (0.8 pmol/µl). Cycle sequencing reactions were performed in the Gene Amp PCR system 9600. Electrophoresis was performed using the 377 Sequencer (ABI,Perkin Elmer, Foster City, CA, USA).

2.1.7. Standard Solutions and Buffers

Acrylamide:Bisacrylamide stock solution (%40)

39.5 acrylamide

0.53g bisacrylamide

The volume was adjusted to 100 ml by adding ddH_2O.

Agarose gel loading buffer (6X)

15 % ficoll

0.05 % bromphenol blue

0.05 % xylene cyanol

Developer Solution

1.5 % NaOH

0.1 % formaldehyde

Extraction buffer

10 mM Tris HCl, pH 8.0

10 mM EDTA, pH 8.0

Proteinase K 20 mg/ml

0.5 % SDS

Fixative Solution

10 % ethanol

0.5 % acetic acid

Silver nitrate solution

0.1% silver nitrate

Sequencing loading buffer

5 parts deionized formamide

1 part EDTA/ blue dextran

25mM EDTA (pH 8.0)

50 mg/ml blue dextran

SSC (20X)

3 M NaCl

0.3 M trisodium citrate, pH 7.0

TE Buffer

10 mM Tris HCl pH 8.0

1 mM EDTA

Tris-boric acid-EDTA (TBE) (10 X) (1L)

108 g Tris HCl

55 g boric acid

20 ml 0.5 M EDTA

q.s. 1000 ml ddH_2O

2.2. Methods

2.2.1. DNA isolation from whole blood specimens

Blood samples have been stored at 4^0C for one to five days. Before starting DNA isolation, blood was frozen in 700 µl aliquots in 1.5 ml eppendorf tubes at -80^0C for at least one day.

Blood was thawed, 800 µl of 1X SSC was added, and the content was mixed by vortexing. Then, it was centrifuged in a microfuge (Heraeus instruments,

Biofuge, Osterode, Germany) at 13,000 rpm for 1 minute. The supernatant was removed without disturbing the cell pellet and discarded into disinfectant. Then 1.4 ml 1X SSC was added and the tube was vortexed briefly to resuspend the cell pellet. Again, it was removed, avoiding the pellet. Cell pellet could be washed several times with 1 X SSC if necessary.

Next, 800 µl extraction buffer (10 mM TrisHCl ph 8.0, 10 mM EDTA pH 8.0, 0.5 % SDS) and 10 µl proteinase K (20 g/ml ddH_2O) were added. The tube was vortexed briefly to resuspend the cell pellet. The suspension was incubated at 56^0C for at least 1 hour. Incubation could be done overnight if necessary to dissolve the cell pellet.

The DNA was then extracted with 400 µl phenol/chloroform/isoamyl alcohol (25:24:1) and vortexed for 60 seconds. This step must be carried out in the fume hood. The tube was spun in a microfuge for 5 minutes at 13,000 rpm. The upper aqueous layer (~ 700 µl) was removed and placed in a new tube. If DNA supernatant was sticky or if the interface was not clear after this step, the supernatant is not removed. An additional extraction step was performed with 350 µl phenol/chloroform/isoamyl alcohol. The recovered supernatant was separated into two or more tubes (350 µl per tube).

The DNA was then precipitated from the suspension by adding 35 µl NaOAc (3M, pH 5.2) and 700 µl ice-cold absolute ethanol (EtOH) were added to each tube, mixing by inversion and placing at - 20^0C for 30 minutes. The tubes were spun in a microfuge for 15 minutes at 13,000 rpm. The alcohol was removed and the pellet

was washed with 1.0 ml room temperature 70 % EtOH. The tubes were spun in a microfuge for 5 minutes at 13,000 rpm. All the alcohol was removed with a micropipette and the tubes were left open on the bench (~30 min) to allow the EtOH to evaporate. The DNA was solubilized in 200 µl TE (pH 8.0) by incubating at 56^0C for at least 1 hour. Incubation was done overnight if necessary to solubilize the pellet. The DNA was then stored at - 20^0C.

The concentration and purity of the double stranded DNA was determined on the Beckman Spectrophotometer Du 640 (Beckman Instruments Inc., Fullerton, CA, USA) using the Beckman Instruments Du Series 600 Spectrophotometer software program. Absorbance readings were taken at wavelengths of 260 nm and 280 nm. The A_{260} allows calculation of the concentration of nucleic acid in the sample. An optical density value of one corresponds to approximately 50 µg/ml of double stranded DNA. The A_{260}/A_{280} ratio provides an estimate of the purity of the nucleic acid. A pure preparation of DNA will have A_{260}/A_{280} ratio between 1.8- 2.0. If there is contamination with protein or phenol, the A_{260}/A_{280} ratio will be significantly less than the values given above and accurate quantitation of the amount of nucleic acid will not be possible.

DNA was also checked by horizontal agarose gel electrophoresis to verify that it was high molecular weight. A 1.0 % agarose minigel with 1 X TBE was prepared. Ethidium bromide (1 µl/ml) was incorporated into the gel. DNA samples were loaded into the sample wells and the gel was run at 80 V. After the run, the DNA was visualized with UV transilluminator.

2.2.2 DNA isolation from hair

Procedures utilizing Chelex 100 chelating resin have been used for extracting DNA from hair samples for use with the polymerase chain reaction. The procedures are simple, rapid and do not involve organic solvents.

Minimum 5-6 individual pieces of rooted hair was pulled out. Then, it was washed with 1-2 ml ddH_2O. Rooted hair was put into an eppendorf tube that included 200 μl 5% chelex. The mixture was incubated overnight at 56^oC. Then vortexed for 10 seconds.

Next boiled for 8 minutes and vortexed for 10 seconds. The tube was spun at 13.000 rpm for 2-3 minutes. Then for PCR reaction, 10-20 μl was taken from supernatant.

2.2.3 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a technique, which is used to amplify the number of copies of a specific region of DNA, in order to produce enough DNA to be adequately tested. There are three distinct events in PCR, which are repeated for 30 to 40 cycles: template denaturation, primer annealing and DNA synthesis. Template DNA is denatured by heating the reaction to $95\text{-}96^0C$. After denaturation, the primers are allowed to hybridize to their complementary single-stranded target sequences. The temperature of this step depends on the homology of the primers for the target sequence as well as the base composition of the oligonucleotides. The last step is the extension of the oligonucleotide primer by the thermostable polymerase.

72^0C is the ideal working temperature for the polymerase. Usually, the larger the template, the longer the time required for a proper extension.

Generally, 50- 100 ng DNA was used as a template. A 25 µl PCR reaction contained 2.5 µl 10 X PCR buffer (final concentration 1 X PCR buffer), 1.5- 3.0 mM $MgCl_2$, 200µM dNTP, 20 pmol forward primer, 20 pmol reverse primer and 1 U Taq polymerase. The volume was adjusted to 25 µl by adding ddH_2O. Setting up a series of PCR reactions using a range of MgCl2 concentrations optimised MgCl2 concentration for each primer pair. Table 6 lists the appropriate $MgCl_2$ concentration and melting temperatures for each exon.

Amplification was performed in the GeneAmp PCR with the following parameters: initial denaturation at 95^0C for 5 min; 30 cycles of 95^0C for 30 sec (denaturation), 57-62^0C for 30 sec (annealing), 72^0C for 30 sec (extension); and a final extension at 72^0C for 10 min. After the PCR cycles were completed, the tubes were held at 4^0C for at least 5 minutes or until removal.

Table 6 Optimum $MgCl_2$ concentrations and T_m for PCR of *MECP2* and *AR* exons

Gene	Exons	Primers	Tm (^{0}C)	[$MgCl_2$] (mM)
MECP2	Exon 3	GA 526/527	62	1.5
	Exon 4.1	GA 530/531	60	1.5
	Exon 4.2	GA 532/533	58	1.5
	Exon 4.3	GA 534/535	62	1
	Exon 4.4	GA 536/537	58	1.5
	Exon 4.5	GA 538/539	60	1.5
AR	Exon 1	GA 542/543	57	1

2.2.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis is a commonly used method for DNA analysis. The method is based on the mobility of DNA molecules in the pores of agarose. Agarose is a chain of sugar molecules, which is extracted from seaweed. DNA has a negative charge in solution, so it will migrate to the positive pole in an electric field. The rate of migration will depend on the amount of charge and on the shape or size of the molecule.

Genomic DNA and PCR products were analysed by using agarose gel electrophoresis. Agarose gels included agarose, 1X TBE and ethidium bromide (20 mg/ml). Runs were performed with 1 X TBE at 90 V for 30 minutes.

2.2.5 Electrophoresis Markers

The length of DNA fragments were estimated by comparing to known molecular weight standards that had been run on the same gel. PUC mix,8 was used as DNA marker. The sizes of the fragments were given in figure 11.

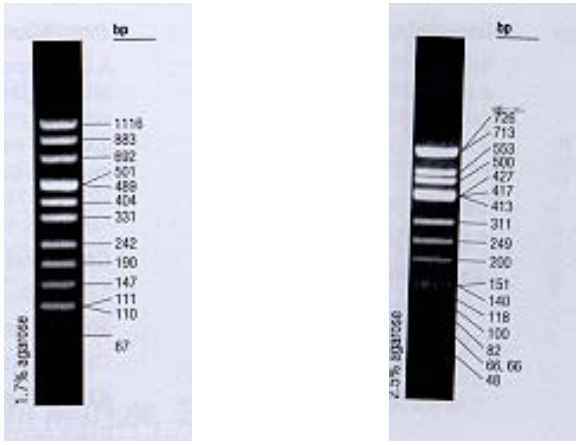


Figure 11. Fragment sizes in pUC mix,8 DNA marker and ϕX174 DNA/HinfI marker,10.

2.2.6 Restriction Enzyme Digestion

Restriction enzyme digestion of PCR products with *Hph*I, *Nla*III, *Nla*IV, *Hha*I, *Hinf*I, *Hpa*II and *Sty*I were performed in 20 µl reaction volumes. Reactions were carried out using the reaction buffer and conditions recommended by the manufacturer. One unit of enzyme was used to digest the PCR products. In order to determine the amount of PCR product that would be used in digestion, the PCR samples were run on agarose gel before the digestion. The incubation temperature was 37^0C for all of the enzymes. After digestion, heat inactivation was performed at 65^0C.

After incubation the cut and uncut PCR fragments were analysed by agarose gel electrophoresis. DNA size markers were used to calculate the sizes of the bands. Electrophoresis was performed using 3 % Nusieve 3:1 agarose or 2 % Metaphor at 5 V/cm in 1X TBE for 2.5 hours. After the electrophoresis, the gel was stained in EtBr (1μg / ml) for 20 minutes and then destained by two 15 minutes washes with distilled water.

2.2.7 Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis is a high-resolution technique. This technique is based on the mobility of DNA molecules from negative pole to positive pole upon voltage application through the porous structure of the polyacrylamide gel.

12% nondenaturing gel was used for analysing the restriction enzyme digestion results. In order to prepare the PAGE, acrylamide / bisacrylamide from 40% stock, 10XTBE was mixed and the volume is completed to 40 ml with ddH_2O. 10% APS and Temed was added to the mixture and poured to the PAGE apparatus. The sample was loaded to the gel and runs are performed at 60 V for 3 hours.

2.2.8 Silver Staining

Silver staining is a method suitable for detection of double-stranded and single-stranded DNA, and is more sensitive than ethidium bromide.

The steps in silver staining was as follows. The gel was rinsed twice in ddH_2O for 1 minute. Then it was incubated in 300 ml fixative solution for 3 minutes

and it was rinsed again in ddH_2O for 1 minute. After this step, the gel was incubated in 300 ml of 0.1% $AgNO_3$ solution for 10 minutes at room temperature and rinsed twice rapidly in ddH_2O. As a last step, 300 ml of ice-cold developer solution was added to the gel and incubated in this solution until the bands develop.

2.2.9 DNA sequence Analysis

The DNA sequencing analysis was performed using the ABI 377 DNA sequencer at Molecular Biology and Genetics Department, Bilkent University. DNA sequence analysis of *MECP2* exon 3 for sample 99-91 using GA 526 and GA 527 primers, exon 4.1 for samples 99-114, 00-132, 00-157 using GA 530 and GA 531 primers, exon 4.3 for samples 99-104, 00-179, 00-187, 00-196 using GA 534 and GA 535 primers were performed. Forward and reverse primer pairs were used for sense strand and antisense strand sequencing. The PCR products were cleaned up with QIAquick PCR purification kit to remove the excess dNTP, $MgCl_2$ etc. The purified PCR products were quantitated via agarose gel electrophoresis by comparing band intensities with a DNA size marker of known concentration. The products with 20-100 ng/µl concentrations were sequenced. For sequencing reactions, Big Dye^{TM} Terminator Cycle Sequencing Ready Reaction Kit (ABI Prism, Catalogue # 4303152) was used. Cycle sequencing reaction was set up as follows; 3-6 µl template, 1 µl primer and 4 µl terminator ready reaction mix was put into 0.2 ml tubes and the volume is adjusted to 20 µl by ddH_2O. The reactions were performed in Perkin Elmer GeneAmp PCR 9600 system according to the following protocol: 25 cycles at 96^0C for 10 sec, at 50^0C for 5 sec, and at 60^0C for 4 min. Sequencing products were precipitated using isopropanol. Then DNA pellet was resuspended in a sequencing loading buffer, and loaded in the 4% sequencing gel.

During electrophoresis, the data was collected using ABI data collection software. After the run, these data were automatically analyzed by an ABI software. Then the files that were analyzed were imported into Factura program to identify the unambigous sequences and subsequently imported to Sequence Navigator program for alignment with the reference DNA sequence.

2.2.10 Allele-specific X-chromosome inactivation assay

For this assay, genomic DNA (1μg) from the patients (who has a mutation) was digested overnight, with methylation sensitive *Hpa*II enzyme, in 25 μl volume, by use of manufacturer's recommended buffer. PCR amplification using GA 542 and 543 primers that amplify 280 bp AR gene was performed. The PCR conditions were; 95^0C for 5 min, followed by 30 cycles at 95^0C for 30 sec, at 56^0C for 30 sec, and at 72^0C 30 sec with a final extension at 72^0C for 10 min. The products were separated on 12% PAGE and then visualized via silver staining.

3. Results

3.1. DNA Isolation

Genomic DNA was isolated from patient's peripheral blood by using a phenol chloroform extraction method (Ausubel et. al, 1994). All extracted DNAs were evaluated quantitatively and qualitatively by UV spectrophotometer, and agarose gel electrophoresis. DNA samples have an optic density ratio of 1.7-1.8. The DNA samples represent single, high molecular weight band on agarose gel (figure 12). The concentrations of these DNA samples are 200, 160, 181 and 140 μg/ml, respectively.

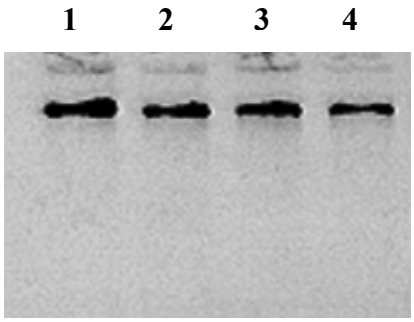


Figure 12. DNA isolation by using phenol/chloroform extraction method

Samples were electrophoresed through a 1% agarose gel at 8V/cm for 35 min.
Lane 1: 01-305; lane 2: 01-320; lane 3: 01-332; lane 4: 01-333

3.2. Polymerase Chain Reaction

All PCR products were analyzed by agarose gel electrophoresis. *MECP2* exon 4.1 primers, GA 530, and GA 531 amplified samples shown in figure 13. The expected size of the PCR product is 380 bp. This was confirmed by comparison of the observed DNA band with the size marker. PCR mixture did not contain contaminating DNA because there was no DNA band in the negative control sample (Appendix 1 shows the schematic representation of the PCR products and primers that were used in the sequence).

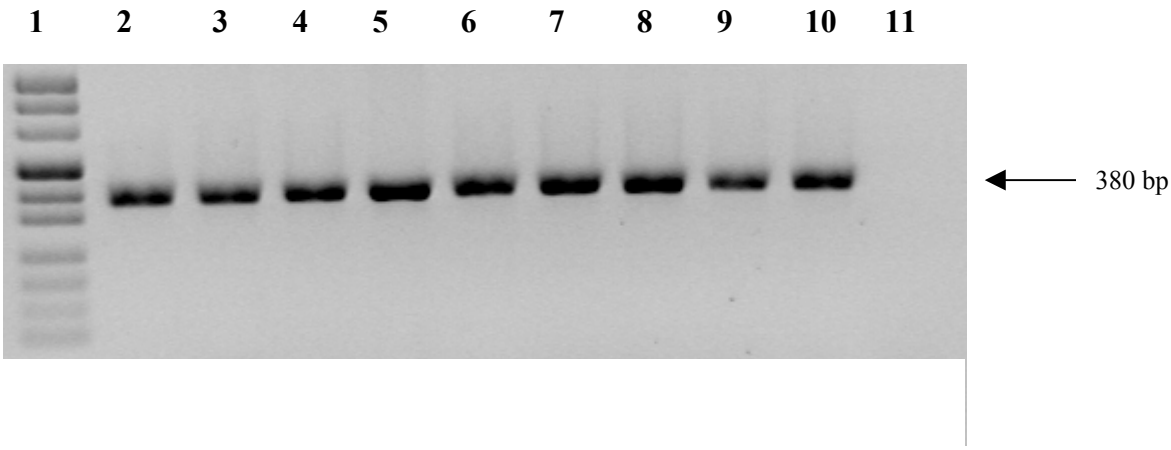


Figure 13. Analysis of PCR products

Samples were electrophoresed through a 2% agarose gel at 8V/cm for 35 min.
Lane 1: pUC mix DNA marker, 8; lanes 2-10: exon 4.1 samples; lane 11: negative control

3.3. Detection of recurrent *MECP2* mutations

Eight different types of mutations, one polymorphism and one deletion were screened. Since, RTT is an X-linked dominant disorder the patients are heterozygous for the mutations. Table 7 lists these mutations and polymorphism (appendix 2 includes a figure that depicts the alterations, that were screened, in the sequence of *MECP2*).

Table 7 *MECP2* mutations and polymorphism that are screened

Alteration Type and Exon	Domain	Nucleotide Change	Amino Acid Change	Restriction Change (+/-)
Missense:				
3	MBD	R106W	316 C→T	NlaIII(+)
4	MBD	P152R	455 C→G	NlaIV(-)
4	MBD	F155S	464 C→T	HinfI(+),TfiI(+)
4	MBD	T158M	473 C→T	NlaIII(+)
4	TRD	R306C	916 C→T	HhaI(-)
Nonsense:				
4	-----	R168X	502 C→T	HphI(+)
4	TRD	R270X	808 C→T	NlaIV(-)
4	TRD	V288X	806 delG	NlaIV(-)
Polymorphism:				
4	-----	E397K	1189 G→A	StyI(+),MnIII(-)

3.3.1. R106W

A C316T point mutation was identified in two affected half sisters (Amir et.al. 1999). This point mutation is a missense mutation that changes Arg to Trp at codon 106 within MBD. The mutation occurs within the recognition site of the restriction enzyme *Nla*III and results in a gain of the cleavage site. Thus, digestion of *MECP2* exon 3 PCR products with *Nla*III might be a simple and cost-effective assay for identifying the other Rett syndrome patients who inherited the same mutation.

*Nla*III has five recognition sites in the wild type *MECP2* exon 3 DNA sequence. GA 526 and GA 527 primer pairs were used in the PCR reaction. The PCR amplified fragment is 597 bp. After *Nla*III digestion the expected fragment

sizes are 156 bp, 152 bp, 141 bp, 67 bp, 50 bp and 31 bp. When there is a R106W mutation, a new *Nla*III cleavage site is created. In a heterozygous individual the expected fragment sizes are 156 bp, 152 bp, 141 bp, 121 bp, 67 bp, 50 bp, 35 bp and 31 bp (schematically depicted in figure 14).

Amplified *MECP2* exon 3 PCR products were incubated with *Nla*III in the recommended buffer at 37^0 C for 3 hours. After digestion, the DNA samples were analysed by polyacrylamide gel electrophoresis.

The *Nla*III digestion result for individual 99-91 is shown in figure 15. Restriction digestion of the 99-91 sample and a control sample with *Nla*III results in a pattern with extra fragments 121 bp and 35 bp (this band is not shown in figure) in lenght, indicating heterozygosity for the R106W mutation in the patient sample, and no extra fragments in the control sample.

DNA samples from 63 RTT patients were analyzed for R106W mutation using this method. Three patients were found to be heterozygous for R106W mutation (99-91, 00-163 and 00-174).

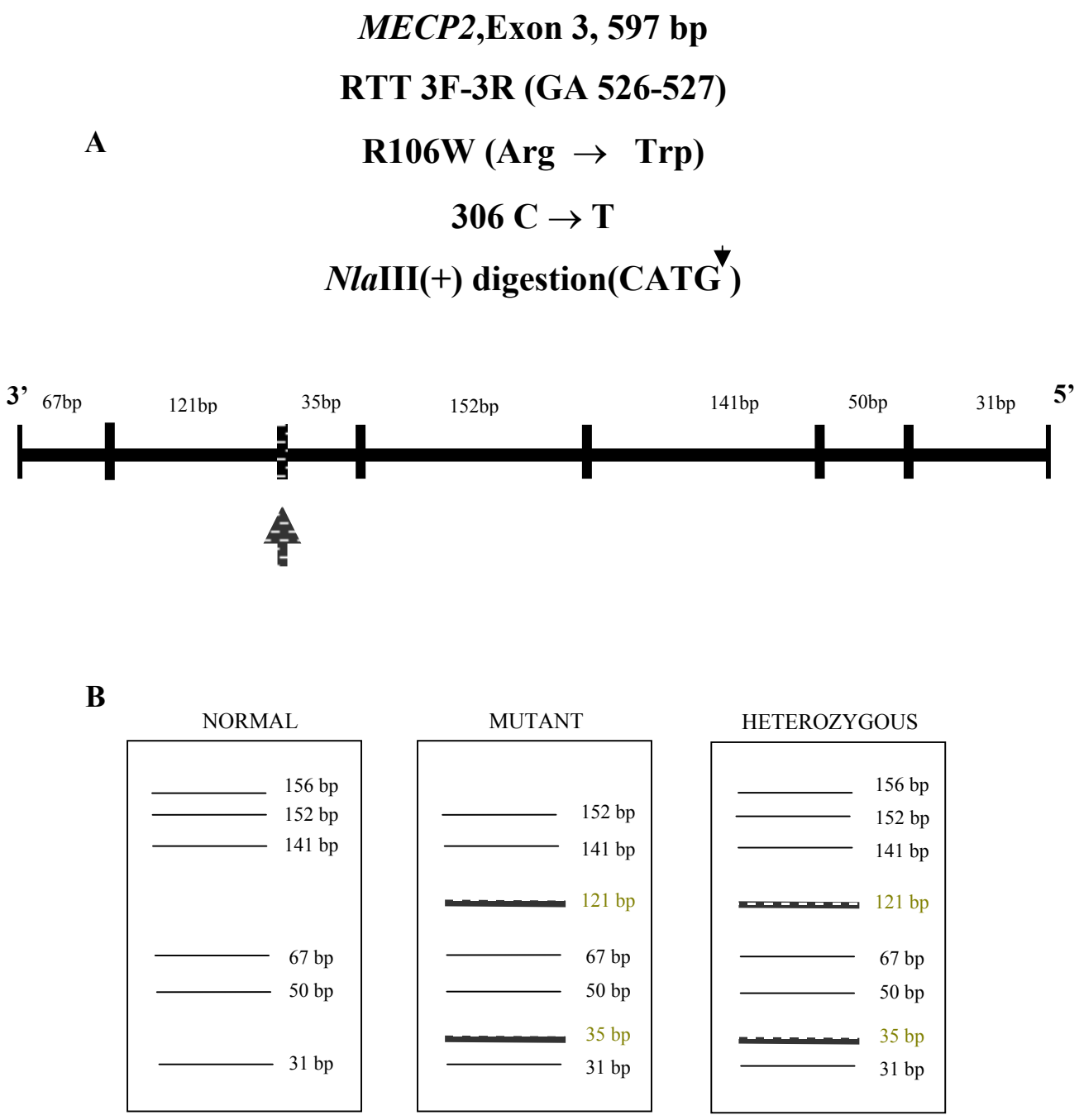


Figure 14. Expected *Nla*III fragment sizes for wild type, mutant and heterozygous individuals.

A. Black lines indicate the recognition / a cleavage site of *Nla*III and the dashed horizontal line indicates the mutation-induced cleavage site of *Nla*III.
B. A schematic representation of the normal and mutant DNA samples run on PAGE is shown.

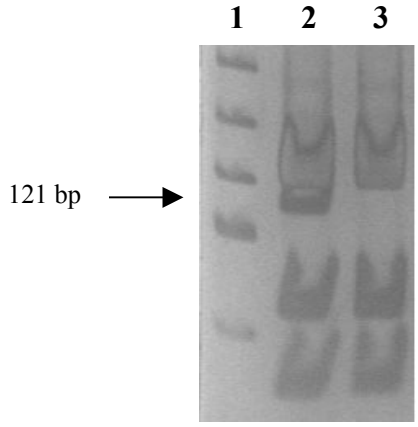


Figure 15. Detection of R106W mutation by cleavage with *Nla*III enzyme.

DNA fragments were electrophoresed in 12% PAGE at 60 V for 4 hours.
Lane 1: pUC mix DNA marker, 8; lane 2: *Nla*III-digested mutant 99-91; lane 3: *Nla*III-digested control.

3.3.2. P152R

A C455G point mutation was identified in one of the sporadic patients (Cheadle et.al. 2000). This point mutation is a missense mutation that changes Pro to Arg at codon 152 within MBD. The mutation occurs within the recognition site of the restriction enzyme *Nla*IV and results in a loss of the cleavage site.

*Nla*IV has four recognition sites in the wild type *MECP2* exon 4.1 DNA sequence. GA 530 and GA 531 primer pairs were used in the PCR reaction. The PCR amplified fragment is 380 bp. When there is a P152R mutation, a *Nla*IV recognition site is abolished. In a heterozygous individual the expected fragment sizes are 213 bp, 175 bp, 95 bp, 49 bp, 38 bp and 23 bp (schematically depicted in figure 16).

Amplified *MECP2* exon 4.1 PCR products were incubated with *Nla*IV in the recommended buffer at 37 ^{0} C for 3 hours. As an uncut control, amplified MECP2 exon 4.1 PCR products were also incubated in the same *Nla*IV buffer, except without the addition of enzyme. After digestion the DNA samples were analysed by agarose gel electrophoresis.

The *Nla*IV digestion result for individual 00-157 is shown in figure 17. Restriction digestion of the 00-157 sample with *Nla*IV results in a pattern with fragments of 213 bp, 95 bp, 49 bp and 23 bp in length, indicating heterozygosity for the F155S mutation and 175 bp, 95 bp, 49 bp, 38 bp and 23 bp fragments in the control sample.

DNA samples from 63 RTT patients were analyzed for F155S mutation using this method. Two patients were heterozygous for F155S mutation (00-133, 00-157).

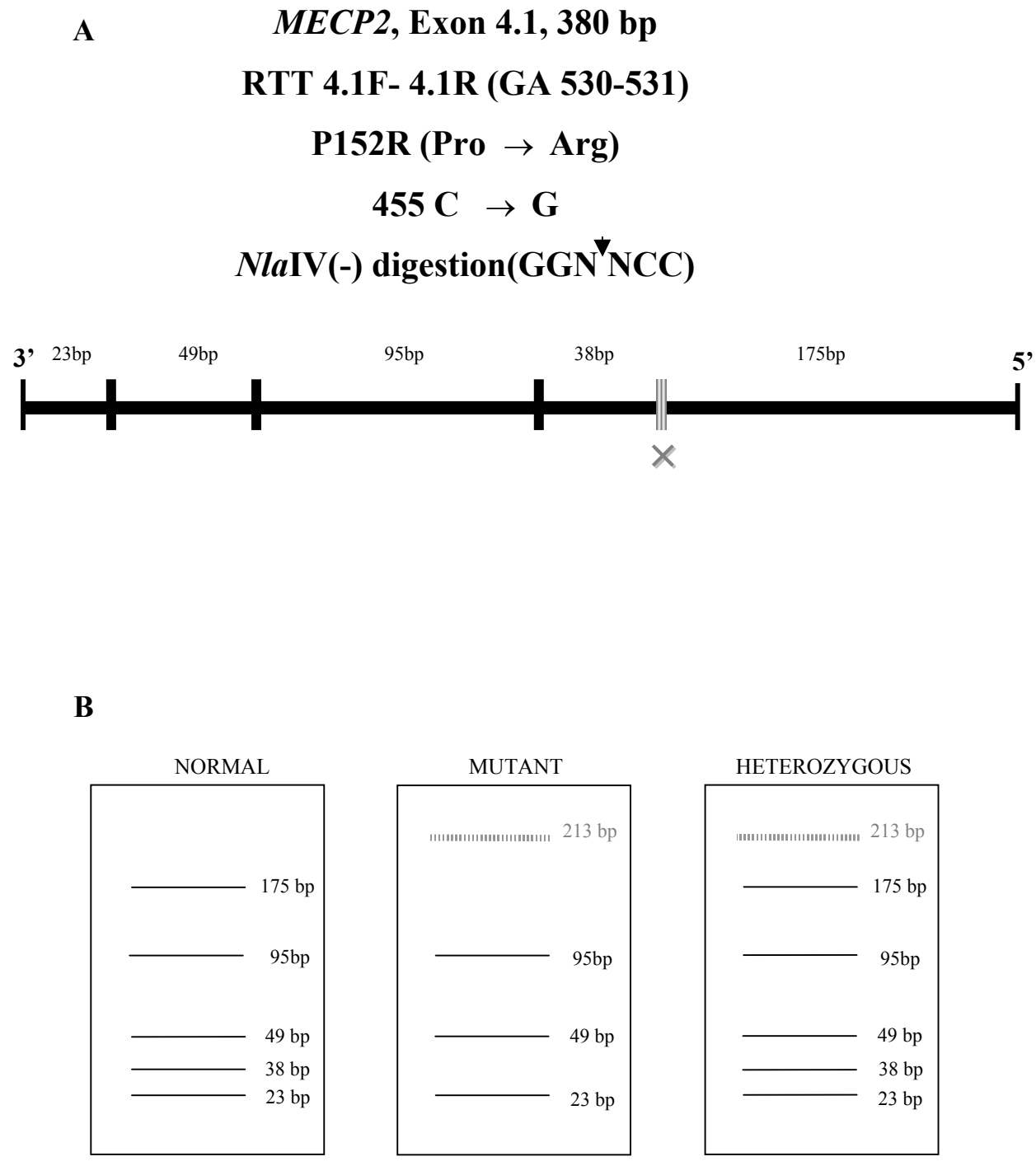


Figure 16. Expected *Nla*IV fragment sizes for wild type, mutant and heterozygous individuals.

A. Black lines indicate the recognition / a cleavage site of *Nla*IV and the narrow vertical line indicates the mutation-induced abolishment of cleavage site of *Nla*IV.
B. A schematic representation of the normal and mutant DNA samples run on agarose gel electrophoresis is shown.

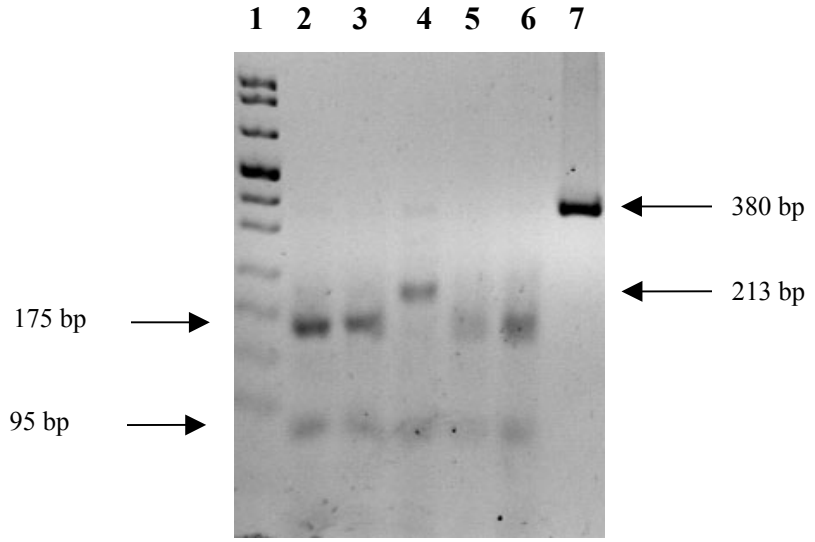


Figure 17. Detection of P152R mutation by cleavage with *Nla*IV enzyme.

DNA fragments were electrophoresed in 2% Metaphor at 60 V for 3 hours.
Lane 1: pUC mix DNA marker, 8; lanes 2-3; 5-6 : *Nla*IV-digested control; lane 4: *Nla*IV-digested mutant 00-157 individual; lane 7: Uncut control

3.3.3. F155S

C464T point mutation was identified in a sporadic case (Amir et.al., 1999). This point mutation is a missense mutation that changes Phe to Ser at codon 155. The mutation occurs within the recognition site of the restriction enzyme *Hinf*I and results in a gain of the restriction site.

*Hinf*I does not have a recognition site in the wild type *MECP2* exon 4.1 DNA sequence. GA 530 and GA 531 primer pairs were used in the PCR reaction. The PCR amplified fragment is 380 bp. When there is a F155S mutation, a *Hinf*I recognition site is created. In a heterozygous individual the expected fragment sizes are 380 bp, 197 bp and 183 bp (schematically depicted in figure 18).

Amplified *MECP2* exon 4.1 PCR products were incubated with *Hinf*I in the recommended buffer at 37 ^{0} C for 3 hours. As *Hinf*I enzyme control, *MECP2* exon 4.4 PCR product were incubated in the recommended buffer at 37 ^{0} C for 3 hours. After digestion, the DNA samples were analysed by polyacrylamide gel electrophoresis.

The *Hinf*I digestion result is shown in figure 19. Restriction digestion of the RTT samples of exon 4.1 result in pattern with 380 bp fragment, indicating that the samples are wild type for F155S mutation. Digestion of the *Hinf*I-digested control of *MECP2* exon 4.4 with the enzyme results in a pattern with fragments 185 bp, 174 bp and 61 bp in length, indicating that enzyme is working.

DNA samples from 63 RTT patients were analyzed for F155S mutation using this method. No F155S mutation was found.

A

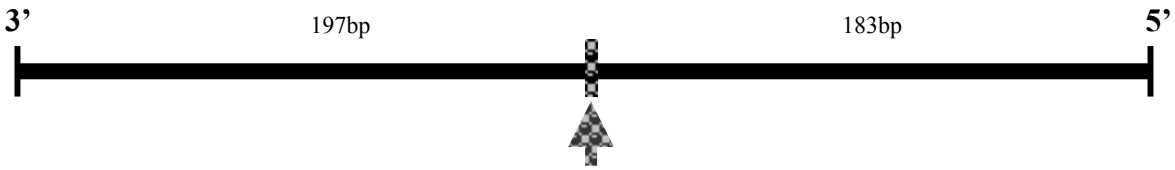
***MECP2*, Exon 4.1, 380 bp**

RTT 4.1F-4.1R (GA 530-531)

F155S (Phe → Ser)

464 C → T

***Hinf*I(+) digestion(G▼ANTC)**



B

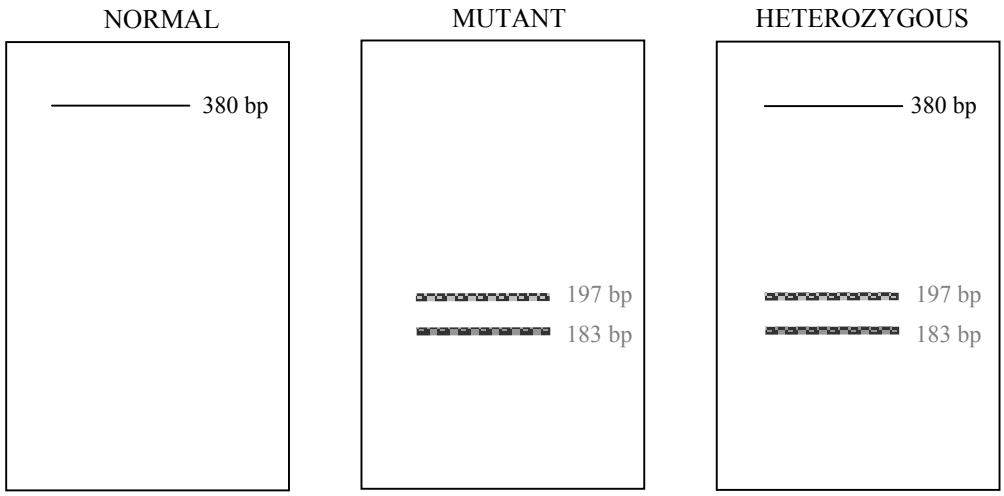


Figure 18. Expected *Hinf*I fragment sizes for wild type, mutant and heterozygous individuals.

A. The sphered line indicates the mutation-induced cleavage site of *Hinf*I.
B. A schematic representation of the normal and mutant DNA samples runs on PAGE is shown.

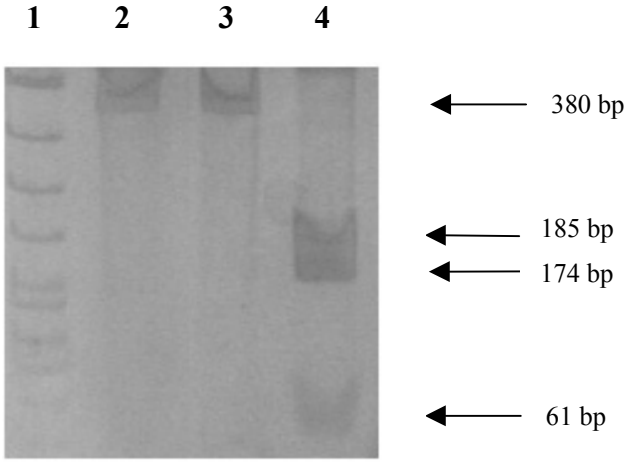


Figure 19. Detection of F155S mutation by cleavage with *Hinf*I enzyme.

DNA fragments were electrophoresed in 12% PAGE at 60 V for 3 hours. Lane 1: ϕX174 DNA marker, 8; lane 2-3: Normal RTT samples (00-163, 00-174); lane 4: *Hinf*I-digested exon 4.4 control (99-95)

3.3.4. T158M

A C473T point mutation was identified in one of the sporadic patients (Amir et.al. 1999). This point mutation is a missense mutation that changes Tyr to Met at codon 158 within MBD. The mutation occurs within the recognition site of the restriction enzyme Nla*III* and results in a gain of the restriction site.

*Nla*III does not have a recognition site in the wild type *MECP2* exon 4.1 DNA sequence. GA 530 and GA 531 primer pairs were used in the PCR reaction. The PCR amplified fragment is 380 bp. When there is a T158M mutation, a *Nla*III recognition site is created. In a heterozygous individual the expected fragment sizes are 380 bp, 197 bp and 183 bp (schematically depicted in figure 20).

Amplified *MECP2* exon 4.1 PCR products were incubated with *Nla*III in the recommended buffer at 37 ^{0} C for 3 hours. After digestion, the DNA samples were analysed by polyacrylamide gel electrophoresis.

The *Nla*III digestion result for individual 99-107 is shown in figure 21. Restriction digestion of the 99-107 sample with *Nla*III results in a pattern with fragments of 380, 197bp and 183 bp in length, indicating heterozygosity for the T158M mutation and a single fragment of 380 bp in the control sample.

DNA samples from 63 RTT patients were analyzed for T158M mutation using this method. Five patients were heterozygous for T158M mutation (99-107, 00-132, 00-155, 00-188, 00-201).

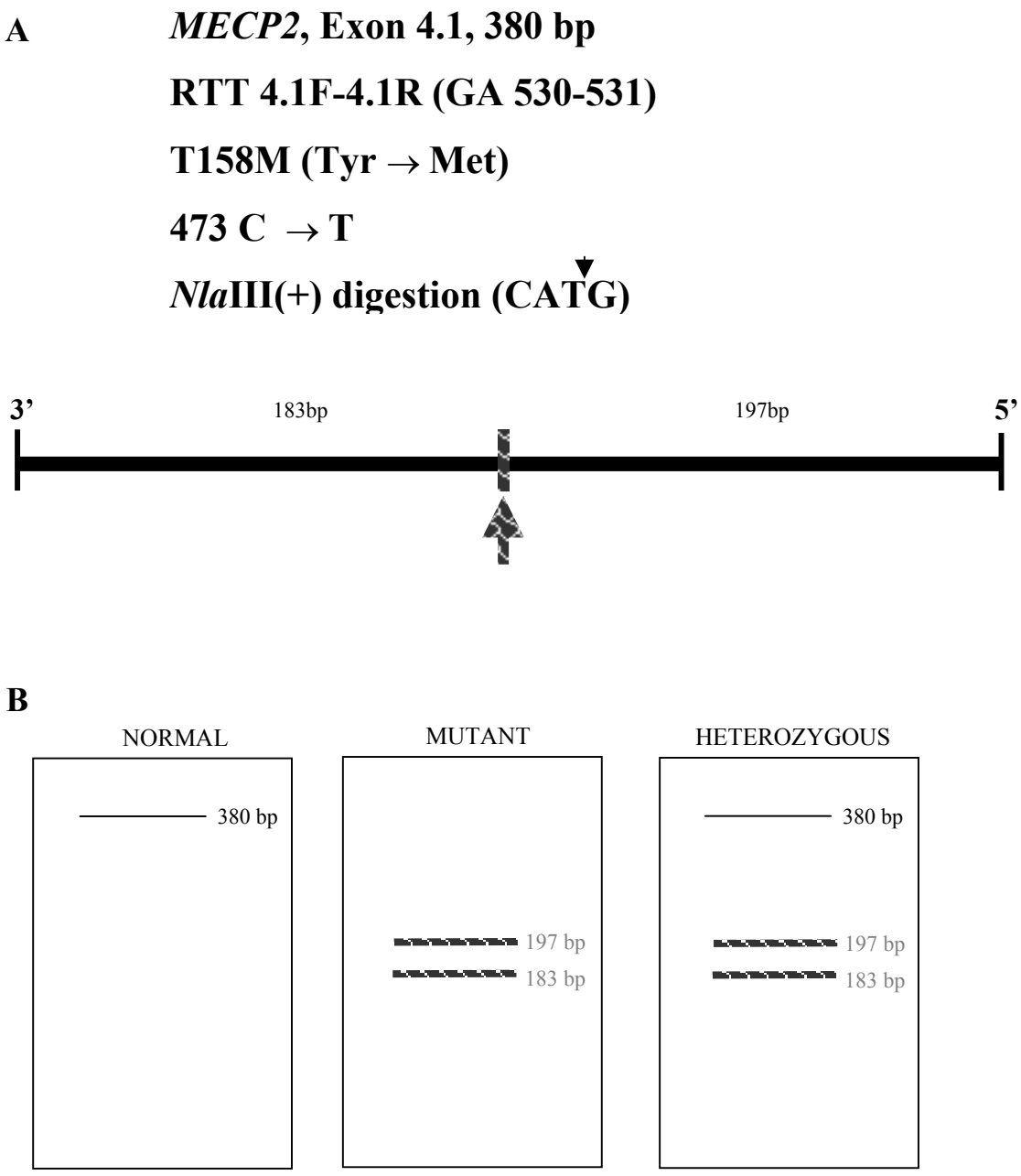


Figure 20. Expected *Nla*III fragment sizes for wild type, mutant and heterozygous individuals.

A. The shingle line indicates the mutation-induced cleavage site of *NlaIII*.
B. A schematic representation of the normal and mutant DNA samples run on PAGE is shown.

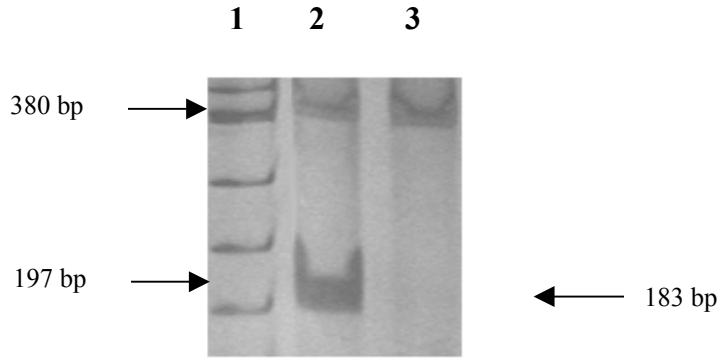


Figure 21. Detection of T158M mutation by cleavage with *Nla*III enzyme.

DNA fragments were electrophoresed in 12% PAGE at 60 V for 4 hours.
Lane 1: ϕX174 DNA marker, 8; lane 2: *Nla*III-digested mutant 99-107; lane 3: *Nla*III-digested control.

3.3.5. R168X

C502T point mutation was identified in six unrelated sporadic cases (Wan et.al. 1999). This point mutation is a nonsense mutation that changes Tyr to stop at codon 168 between the MBD and TRD. The mutation occurs within the recognition site of the restriction enzyme *Hph*I and results in a gain of the restriction site.

*Hph*I does not have a recognition site in the wild type *MECP2* exon 4.1 DNA sequence. GA 530 and GA 531 primer pairs were used in the PCR reaction. The PCR amplified fragment is 380 bp. When there is a R168X mutation, a *Hph*I recognition site is created. In a heterozygous individual the expected fragment sizes are 358 bp, 235bp, 123 bp and 22 bp (schematically depicted in figure 22).

Amplified *MECP2* exon 4.1 PCR products were incubated with *Hph*I in the recommended buffer at 37 ^{0}C for 3 hours. As an uncut control, amplified MECP2 exon 4.1 PCR products were also incubated in the same *Hph*I buffer, except without the addition of enzyme. As *Hph*I enzyme control, *hMLH1* exon 14 PCR product were incubated in the recommended buffer at 37 ^{0}C for 3 hours. After digestion, the DNA samples were analysed by agarose gel electrophoresis.

The *HphI* digestion result is shown in figure 23. Restriction digestion of the RTT samples results in pattern with fragments 358 bp and 22 bp (not shown in figure), indicating that the samples are wild type for R168X mutation.Digestion of the *Hph*I-digested control of *hMLH1* exon 14 with the enzyme results in a pattern with fragments 254 bp, 183 bp and 71 bp in length, indicating that enzyme is working.

DNA samples from 63 RTT patients were analyzed for R168X mutation using this method. No R168X mutation was found.

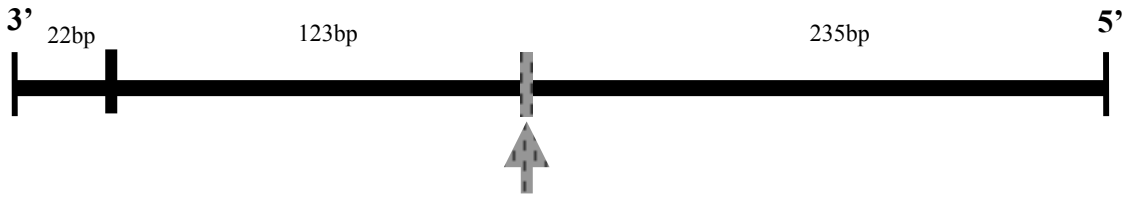
A ***MECP2*,Exon 4.1, 380 bp**

RTT 4.1F-4.1R (GA 530-531)

R168X (Arg → Stop)

502 C → T

***Hph*I(+) digestion(GGTGA(N)$_8$ ▼)**



B

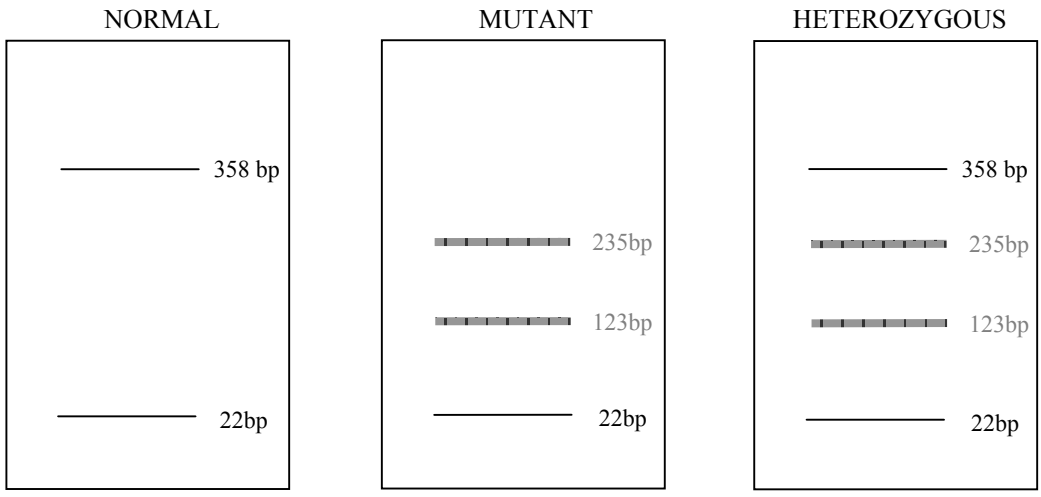


Figure 22. Expected *Hph*I fragment sizes for wild type, mutant and heterozygous individuals.

A. Black lines indicate the recognition / a cleavage site of *Hph*I and the dashed vertical line indicates the mutation-induced cleavage site of *Hph*I.
B. A schematic representation of the normal and mutant DNA samples run on agarose gel electrophoresis is shown.

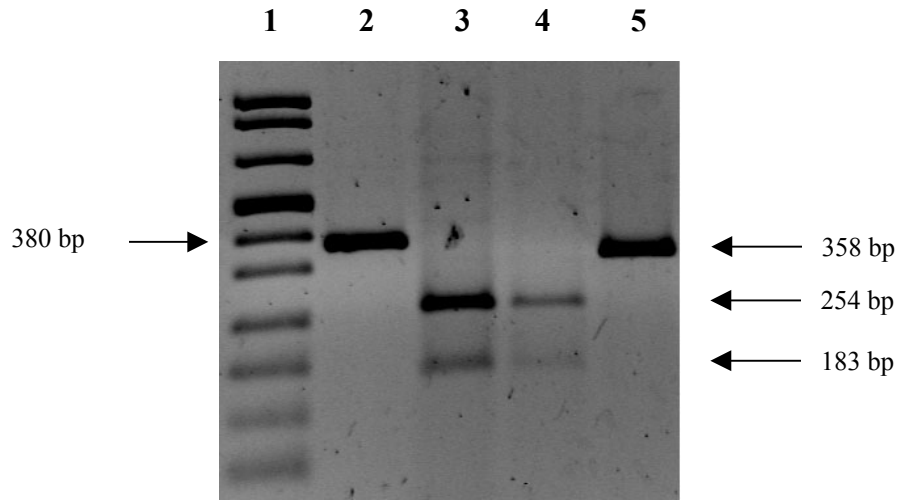


Figure 23. Detection of R168X mutation by cleavage with *Hph*I enzyme.

DNA fragments were electrophoresed in 4% NuSieve at 60 V for 3 hours.
Lane 1: pUC mix DNA marker, 8; lane 2: Uncut control; lane 3-4: *Hph*I-digested *hMLH1* exon 14 control (97-2,97-168); lane 5: RTT sample (00-150)

3.3.6. R306C

A C916T point mutation was identified in one of the sporadic patients. This mutation is the first missense mutation identified in TRD (Wan et.al. 1999). This point mutation changes Arg to Cys at codon 306. The mutation occurs within the recognition site of the restriction enzyme HhaI and results in a loss of the cleavage site.

*Hha*I has three recognition sites in the wild type *MECP2* exon 4.3 DNA sequence. GA 534 and GA 535 primer pairs were used in the PCR reaction. The PCR amplified fragment is 366 bp. When there is a R306C mutation, a *Hha*I recognition site is abolished. In a heterozygous individual the expected fragment sizes are 308 bp, 164bp, 144 bp, 47 bp and 11 bp (schematically depicted in figure 24).

Amplified *MECP2* exon 4.3 PCR products were incubated with *Hha*I in the recommended buffer at 37 ^{0} C for 3 hours. As an uncut control, amplified MECP2 exon 4.3 PCR products were also incubated in the same *Hha*I buffer, except without the addition of enzyme. After digestion the DNA samples were analysed by agarose gel electrophoresis.

The *Hha*I digestion result for individual 00-179 is shown in figure 25. Restriction digestion of the 00-179 sample with *Hha*I results in a pattern with fragments of 308 bp, 164 bp, 144 bp, 47 bp and 11 bp in length, indicating heterozygosity for the R306C mutation and 164 bp, 144 bp, 47 bp and 11 bp fragments in the control sample.

DNA samples from 63 RTT patients were analyzed for R306C mutation using this method. Five patients were heterozygous for R306C mutation (99-95,00-43,00-44,00-160,00-179).

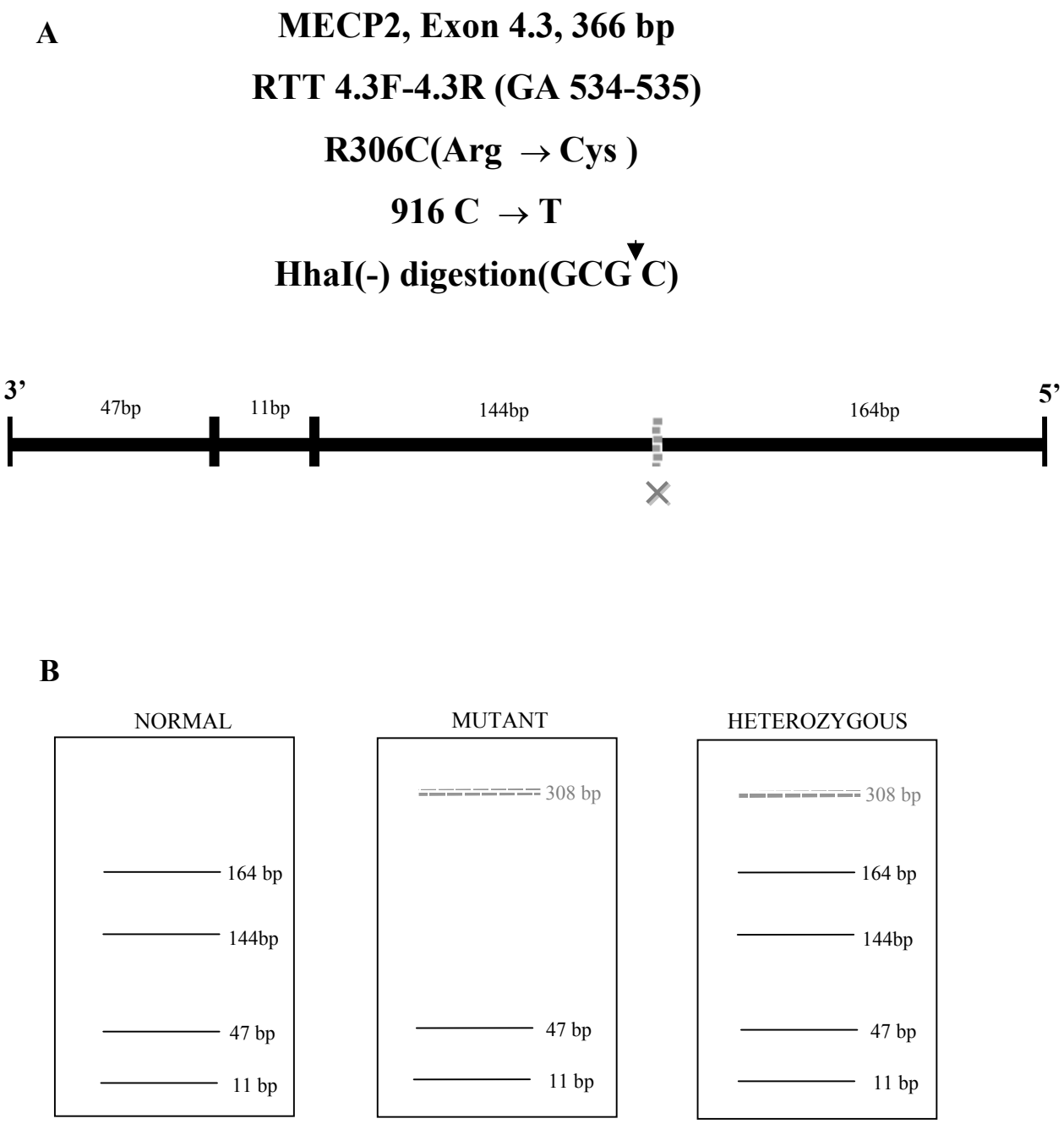


Figure 24. Expected *Hha*I fragment sizes for wild type, mutant and heterozygous individuals.

A. Black lines indicate the recognition / a cleavage site of HhaI and the horizantal brick line indicates the mutation-induced abolishment of cleavage site of *Hha*I.
B. A schematic representation of the normal and mutant DNA samples run on agarose gel electrophoresis is shown.

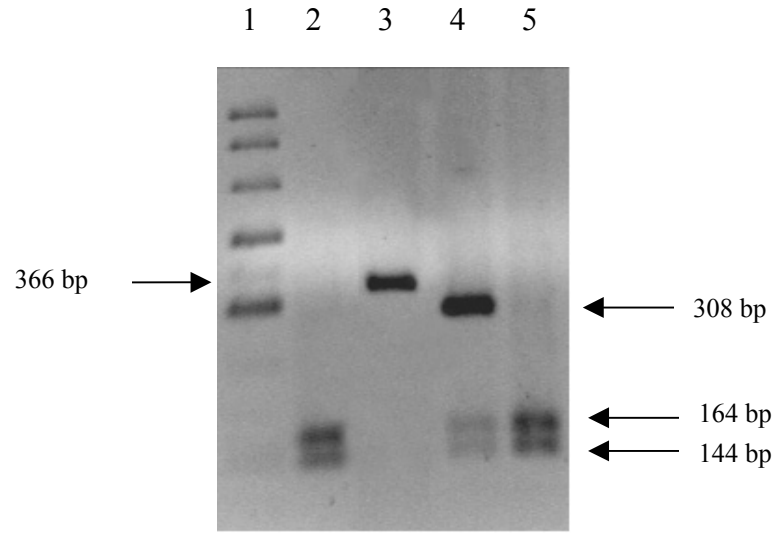


Figure 25. Detection of R306C mutation by cleavage with *Hha*I enzyme.

DNA fragments were electrophoresed in 2% Metaphor at 60 V for 3 hours.
Lane 1: pUC mix DNA marker, 8; lanes 2 and 5: *Hha*I-digested control; lane 3: Uncut control; lane 4: *Hha*I-digested mutant 00-179 individual.

3.3.7. R270X/V288X

C808T point mutation was identified in three sporadic cases (Cheadle et.al., 2000) and del806G was identified in all affected members of a two-generation family (Wan *et al.*, 1999). C808T point mutation is a nonsense mutation that changes Arg to stop at codon 270 within TRD. Del806G causes a frameshift mutation, after 19 missense amino acids, leads to a stop codon at position 288 within the TRD. These two mutations occur within the recognition site of the restriction enzyme *Nla*IV and results in a loss of the restriction site.

*Nla*IV have a recognition site in the wild type *MECP2* exon 4.3 DNA sequence. GA 534 and GA 535 primer pairs were used in the PCR reaction. The PCR amplified fragment is 366 bp. When there is an R270X or V288X mutation, this *Nla*IV recognition site is abolished. In a heterozygous individual with R270X

mutation, the expected fragment sizes are 366 bp, 314 bp and 52 bp and for V288X mutation in heterozygous individual, the expected fragment sizes are 365 bp, 314 bp and 52 bp (schematically depicted in figure 26).

Amplified *MECP2* exon 4.3 PCR products were incubated with *Nla*IV in the recommended buffer at 37 ^{0} C for 3 hours. As an uncut control, amplified MECP2 exon 4.3 PCR products were also incubated in the same *Nla*IV buffer, except without the addition of enzyme. After digestion, the DNA samples were analysed by agarose gel electrophoresis.

The *Nla*IV digestion result is shown in figure 27. Restriction digestion of the RTT samples results in pattern with fragments 366/365 bp, 314 bp and 52 bp (not shown in figure), indicating that the samples are heterozygous for the R270X or V288X mutation.

DNA samples from 63 RTT patients were analyzed for the R270X/V288X mutations using this method. Three patients were found to have a mutation. In order to identify whether this mutation was R270X or V288X, DNA sequencing was performed. According to the result of the sequencing reactions, these three patients had the R270X mutation (99-104, 00-187, 00-196).

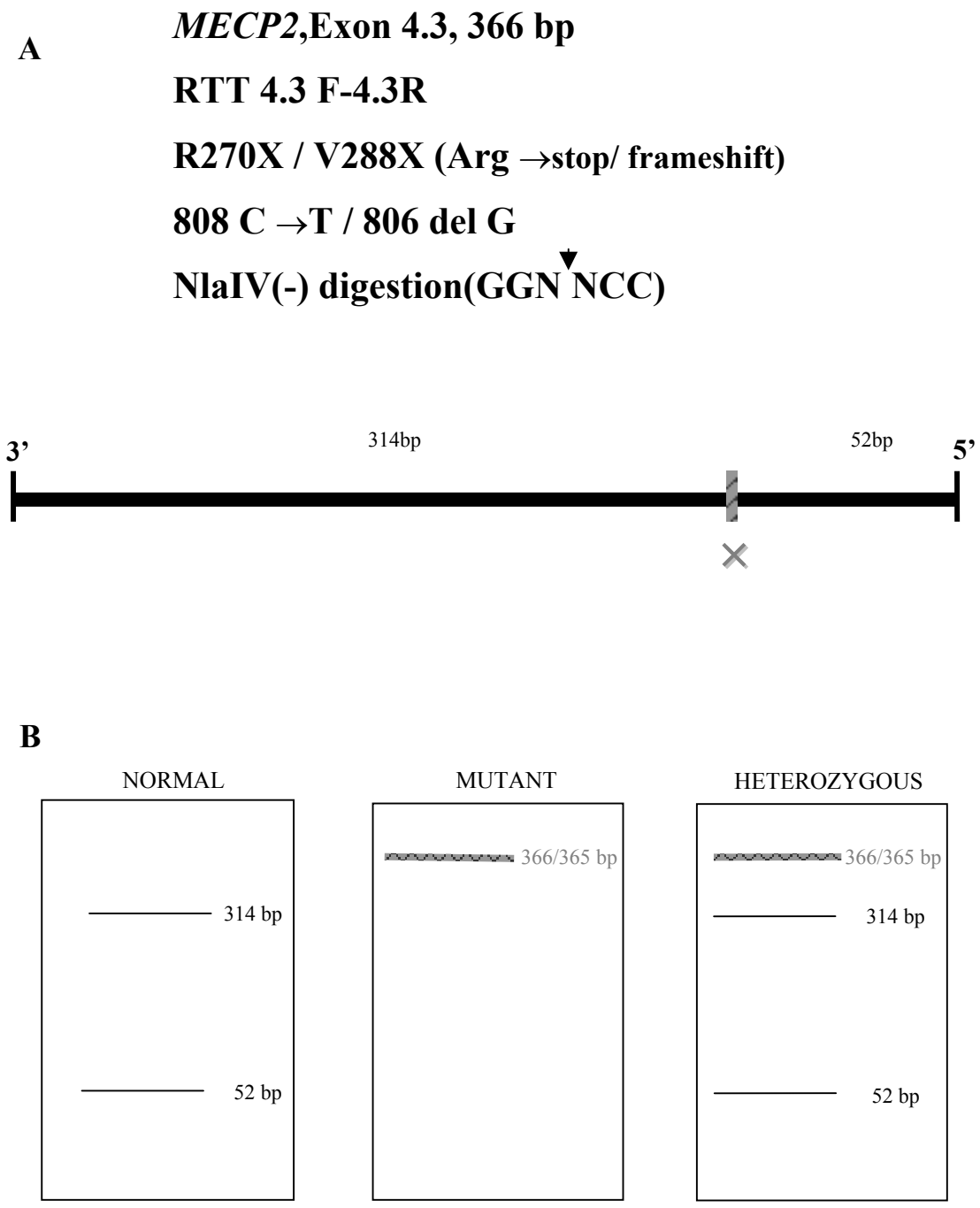


Figure 26. Expected *Nla*IV fragment sizes for wild type, mutant and heterozygous individuals.

A. The diagonal brick line indicates the mutation-induced abolishment of cleavage site of *Nla*IV.
B. A schematic representation of the normal and mutant DNA samples run on agarose gel electrophoresis is shown.

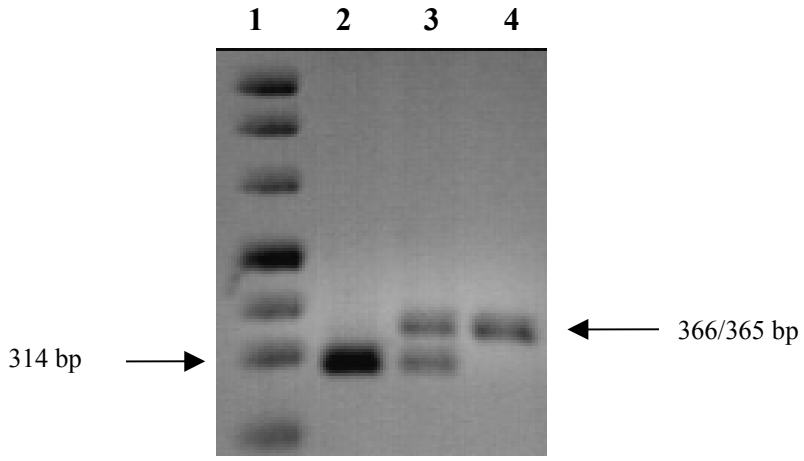


Figure 27. Detection of R270X/V288X mutation by cleavage with *Nla*IV enzyme.
DNA fragments were electrophoresed in 2% Metaphor at 60 V for 3 hours.
Lane 1: pUC mix DNA marker, 8; lane 2: *Nla*IV-digested control; lane 3: *Nla*IV-digested mutant 99-104 individual, lane 4: Uncut control

3.4 Detection of E397K *MECP2* polymorphism by *Sty*I

A C1189T polymorphism was identified in two unrelated families (Wan et.al. 1999). This polymorphism changes Glu to Lys at codon 397. The alteration occurs within the recognition site of the restriction enzyme *Sty*I and results in a gain of the cleavage site.

*Sty*I has two recognition sites in the wild type *MECP2* exon 4.4 DNA sequence. GA 536 and GA 537 primer pairs were used in the PCR reaction. The PCR amplified fragment is 414 bp. When there is a E397K polymorphism, a *Sty*I recognition site is created. In a heterozygous individual the expected fragment sizes are 237 bp, 135bp, 102 bp, 101 bp and 76 bp (schematically depicted in figure 28).

Amplified *MECP2* exon 4.4 PCR products were incubated with *Sty*I in the recommended buffer at 37 ^{0} C for 3 hours. After digestion the DNA samples were analysed by polyacrylamide gel electrophoresis.

The *Sty*I digestion result for individual 00-160 is shown in figure 29. Restriction digestion of the 00-160 sample with *Sty*I results in a pattern with fragments of 237 bp, 135bp, 102 bp, 101 bp and 76 bp in length, indicating heterozygosity for the E397K polymorphism and 237 bp, 101 bp and 76 bp fragments in the control sample.

DNA samples from 60 RTT patients were analyzed for E397K polymorphism using this method. One patient who also bears R306C mutation was heterozygous for E397K polymorphism (00-160).

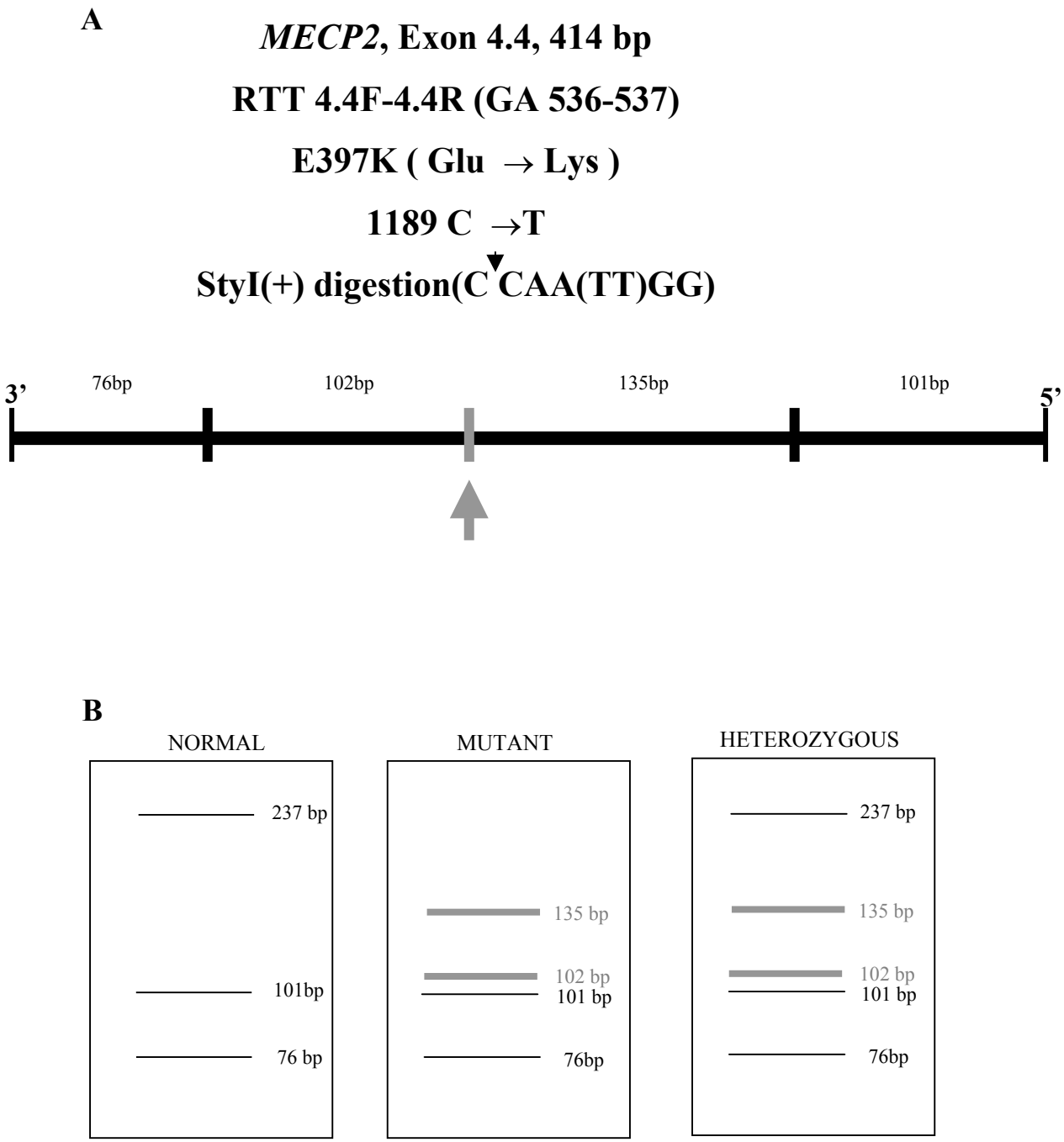


Figure 28. Expected *Sty*I fragment sizes for wild type, mutant and heterozygous individuals.

A. The grey line indicates the mutation-induced cleavage site of *Sty*I.
B. A schematic representation of the normal and mutant DNA samples run on PAGE is shown.

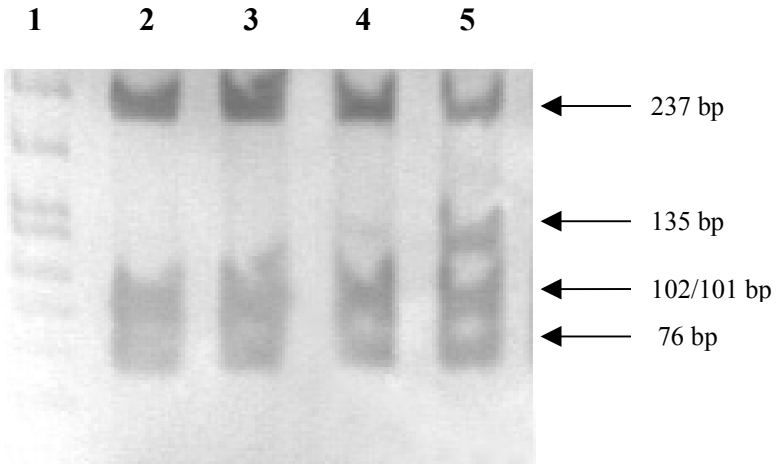


Figure 29. Detection of E397K polymorphism by cleavage with *Sty*I enzyme.

DNA fragments were electrophoresed in 12% PAGE at 60 V for 4 hours.
Lane 1: pUC mix DNA marker, 8; lane 2-4: *Sty*I-digested control; lane 5: *Sty*I-digested 00-160 individual.

Totally, 18 mutations and 1 polymorphism were found in 69 RTT patients. Table 8, indicates the samples which were found to have *MECP2* mutations and polymorphism.

Table 8 RTT patients with *MECP2* alterations

MECP2 ALTERATIONS						
Alteration Type and Exon	**Domain**	**Nucleotide Change**	**Amino Acid Change**	**Restriction Change(+/-)**	**Frequency**	**Samples**
Missense:						
2	MBD	316C→T	R106W	NlaIII (+)	3 / 63	99-91,00-163,00-174
3	MBD	455C→G	P152R	NlaIV (-)	2 / 63	00-133,00-157
3	MBD	464C→T	F155S	HinfI (+),TfiI (+)	0 / 63	---------
3	MBD	473C→T	T158M	NlaIII (+)	5 / 63	99-107,00-132,00-155,00-188,00-201
3	TRD	916C→T	R306C	HhaI (-)	5 / 63	99-95,00-43,00-44,00-160,00-179
Nonsense:						
3	.....	502C→T	R168X	HphI (+)	0 / 63	---------
3	TRD	808C→T	R270X	NlaIV (-)	3 / 63	99-104,00-187,00-196
3	TRD	806del→G	V288X	NlaIV (-)	0 / 63	---------
Polymorphism:						
3	.....	1189C→T	E397K	StyI (+),MnlII (-)	1 / 60	00-160

3.5. Detection of 3' deletion by PCR-based approach

For RTT patients, 00-45 and 00-184, after the PCR amplification of *MECP2* exon 4.4 by the primers GA 536 and GA 537 there was a visible doublet on acrylamide gel (figure 30). Between the two bands in the gel, 44 bp difference is calculated. In that region, Buyse *et al.* 2000, reported a 44 bp deletion, 1164del44nt and recently Nielsen *et al.* 2001, reported another 44 bp deletion, 1155 del 44nt, so that, 00-45 and 00-184 was proposed to have 1164del44nt, 1155 del44nt or another deletion with 44 bp. Consequently, it needs to be analyzed subsequently by subcloning and sequencing to indicate the type of deletion.

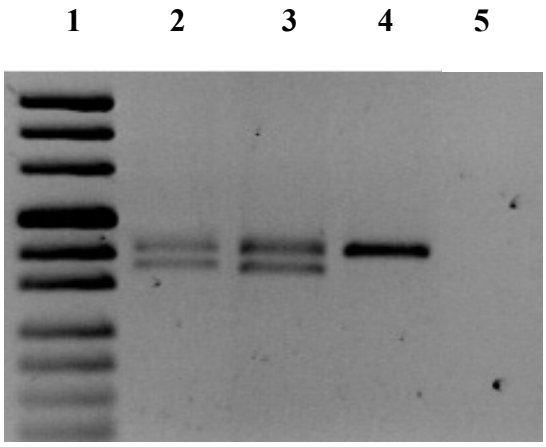


Figure 30. Detection of 3' deletion at PCR level

Lane 1: pUC mix, DNA marker, 8; lane 2: 00-45; lane 3: 00-184, lane 4: 00-174, lane 5: negative control

3.6. Detection of unknown mutation

During the detection of T158M mutation via *Nla*III enzyme digestion, 99-114 sample displayed different pattern (figure 31). There was ~ 310 bp extra band, probably because of the creation of new recognition site for *Nla*III. Subsequent analysis by sequencing indicated that there was C590T transition, which leaded to T197M mutation in 99-114 individual (figure 36).

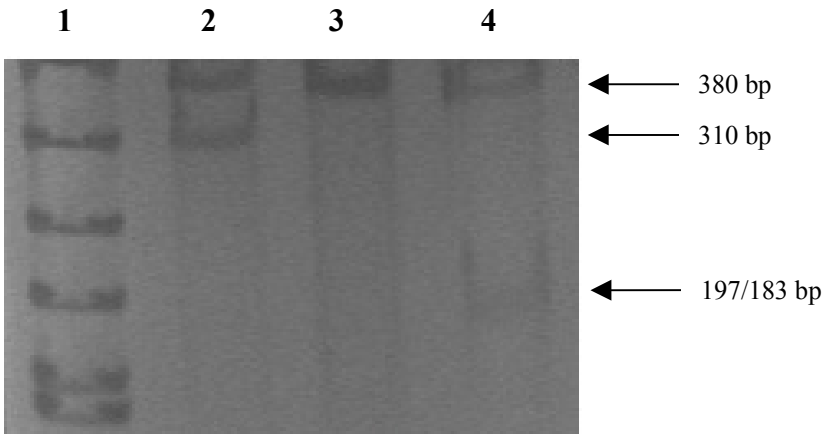


Figure 31. Detection of unknown mutation by cleavage with *Nla*III enzyme.

DNA fragments were electrophoresed in 12% PAGE at 60 V for 4 hours.
Lane 1: pUC mix DNA marker, 8; lane 2: *Nla*III-digested 99-114; lane 3: *Nla*III-digested control, lane 4: *Nla*III-digested 00-155 with T158M mutation.

3.7. DNA Sequence Analysis

3.7.1. *MECP2* exon 3 R106W mutation

Cycle sequencing reactions were performed using *MECP2* exon 3 primers, GA 526 and GA 527 and ABI prism kit, for 99-91 sample in order to confirm that the template had R106W mutation. By using ABI Sequence Analysis Software on the 377 DNA Sequencer, the DNA products were electrophoresed and analyzed. Heterozygous bases were identified using the Factura software program. Then, finally the DNA sequence was aligned with the reference sequence using the Sequence Navigator Software Program. The only difference between the reference sequence and 99-91 sample was at nucleotide 316 in exon 3. At this position, although there was C (cytosine) residue in the reference sequence, the 99-91 sample was heterozygous "N" with one allele having a C (cytosine) residue and the other allele having a T (tyrosine) residue at this point (figure 32). Thus, after restriction enzyme digestion results, it was confirmed by sequencing that 99-91, 00-163, 00-174 samples had R106W mutation.

a

b

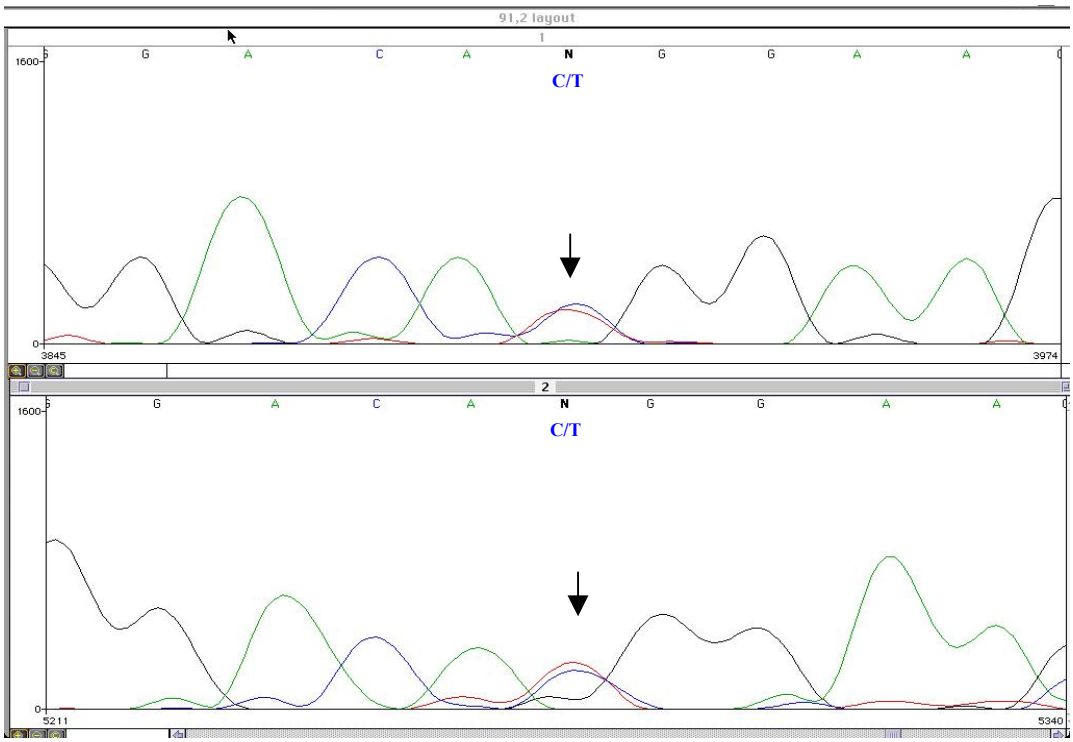


Figure 32. Electropherogram showing R106W (306 C to T) mutation.

a. Sample 99-91: DNA sequence obtained with forward primer,
b. Sample 99-91: DNA sequence obtained with reverse primer.

3.7.2. *MECP2* exon 4.1 P152R mutation

Cycle sequencing reactions were performed using *MECP2* exon 4.1 primers, GA 530 and GA 531 and ABI prism kit, in order to confirm the RE digestion result for P152R mutation. One of the sample found to be mutant by RE digestion, 00-133, was sequenced. By using ABI Sequence Analysis Software on the 377 DNA Sequencer, the DNA products were electrophoresed and analyzed. Heterozygous bases were identified using the Factura software program. Then, finally the DNA sequence was aligned with the reference sequence using the Sequence Navigator Software Program. The only difference between the reference sequence and 00-133 sample was at nucleotide 455 in exon 4.1. At this position, although there was C (cytosine) residue in the reference sequence, the 00-133 sample was heterozygous

"N" with one allele having a C (cytosine) residue and the other allele having a G (guanine) residue at this point (figure 33).

a

b

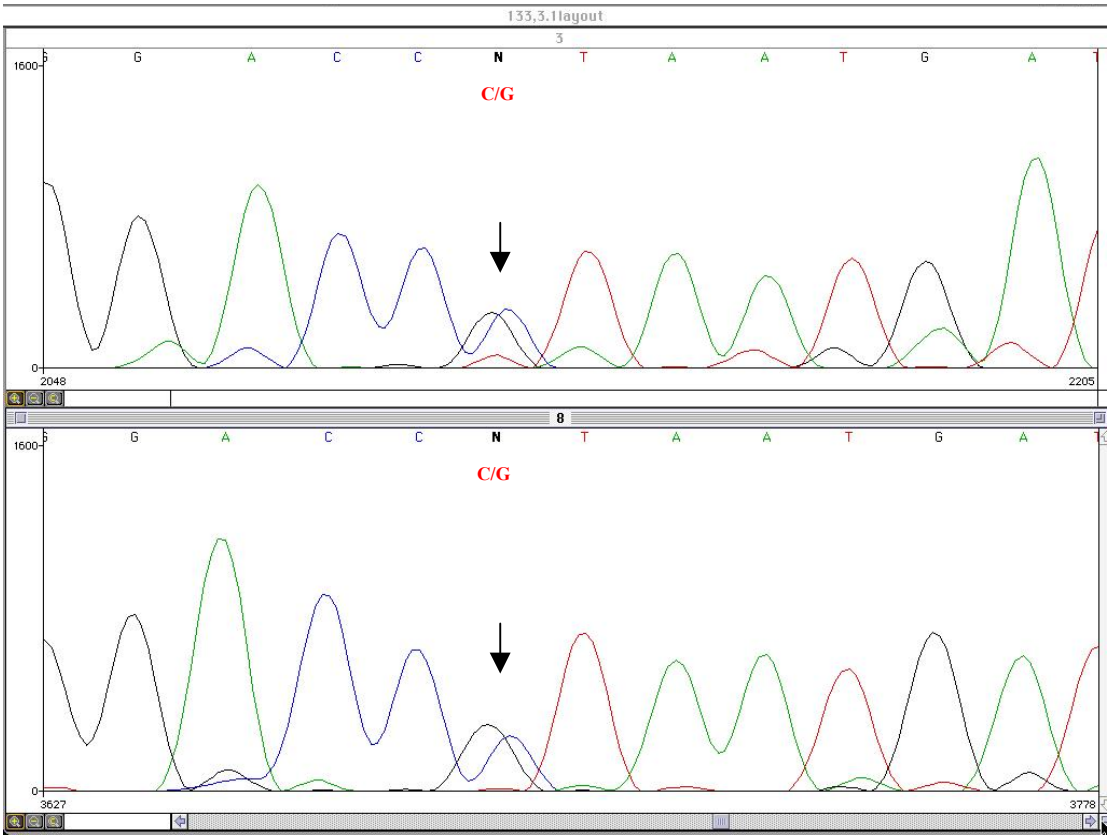


Figure 33. Electropherogram showing P152R (455 C to G) mutation.

a. Sample 00-133: DNA sequence obtained with forward primer,
b. Sample 00-133: DNA sequence obtained with reverse primer.

3.7.3. *MECP2* exon 4.1 T158M mutation

Cycle sequencing reactions were performed using *MECP2* exon 4.1 primers, GA 530 and GA 531 and ABI prism kit, in order to confirm the RE digestion result for T158M mutation. One of the sample found to be mutant by RE digestion, 00-132, was sequenced. By using ABI Sequence Analysis Software on the 377 DNA Sequencer, the DNA products were electrophoresed and analyzed. Heterozygous bases were identified using the Factura software program. Then, finally the DNA sequence was aligned with the reference sequence using the Sequence Navigator

Software Program. The only difference between the reference sequence and 00-132 sample was at nucleotide 473 in exon 4.1. At this position, although there was C (cytosine) residue in the reference sequence, the 00-132 sample was heterozygous "N" with one allele having a C (cytosine) residue and the other allele having a T (tyrosine) residue at this point (figure 34).

a

b

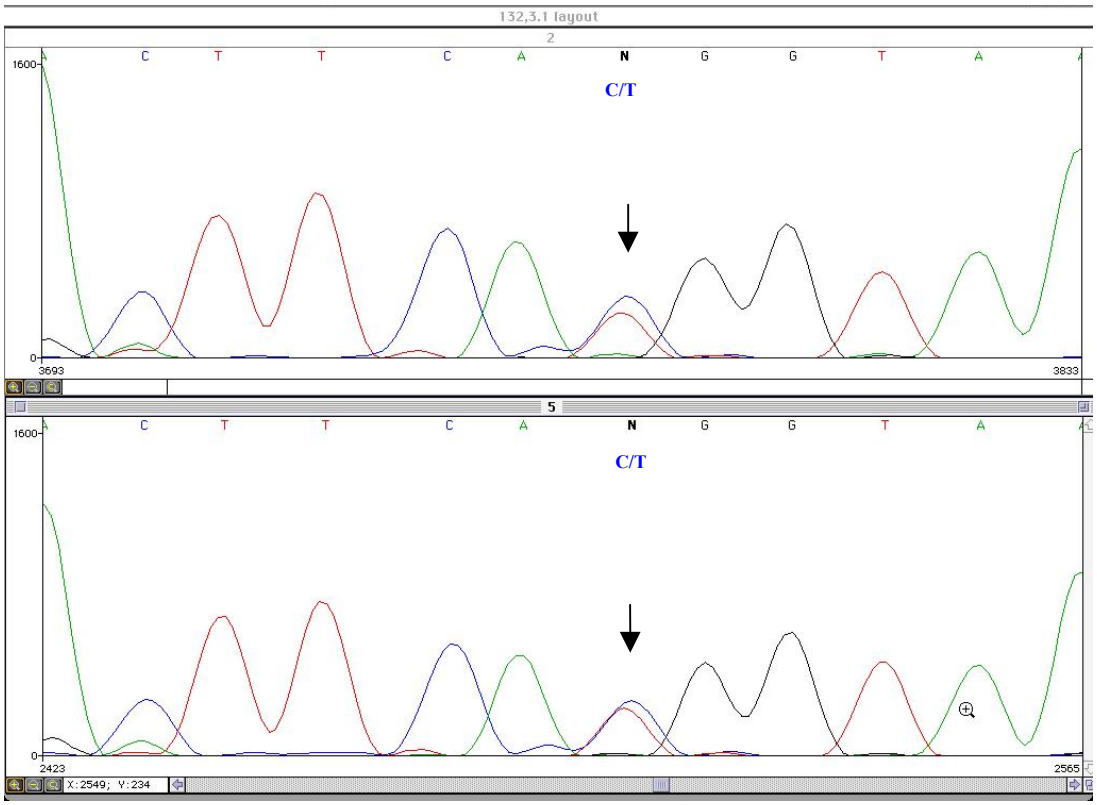


Figure 34. Electropherogram showing T158M (473 C to T) mutation.

a. Sample 00-132: DNA sequence obtained with forward primer,
b. Sample 00-132: DNA sequence obtained with reverse primer.

3.7.4. *MECP2* exon 4.3 R306C mutation

Cycle sequencing reactions were performed using *MECP2* exon 4.3 primers, GA 534 and GA 535 and ABI prism kit, in order to confirm the RE digestion result for R306CM mutation. One of the sample found to be mutant by RE digestion,

00-179, was sequenced. By using ABI Sequence Analysis Software on the 377 DNA Sequencer, the DNA products were electrophoresed and analyzed. Heterozygous bases were identified using the Factura software program. Then, finally the DNA sequence was aligned with the reference sequence using the Sequence Navigator Software Program. The only difference between the reference sequence and 00-179 sample was at nucleotide 916 in exon 4.3. At this position, although there was C (cytosine) residue in the reference sequence, the 00-179 sample was heterozygous "N" with one allele having a C (cytosine) residue and the other allele having a T (tyrosine) residue at this point (figure 35).

a

b

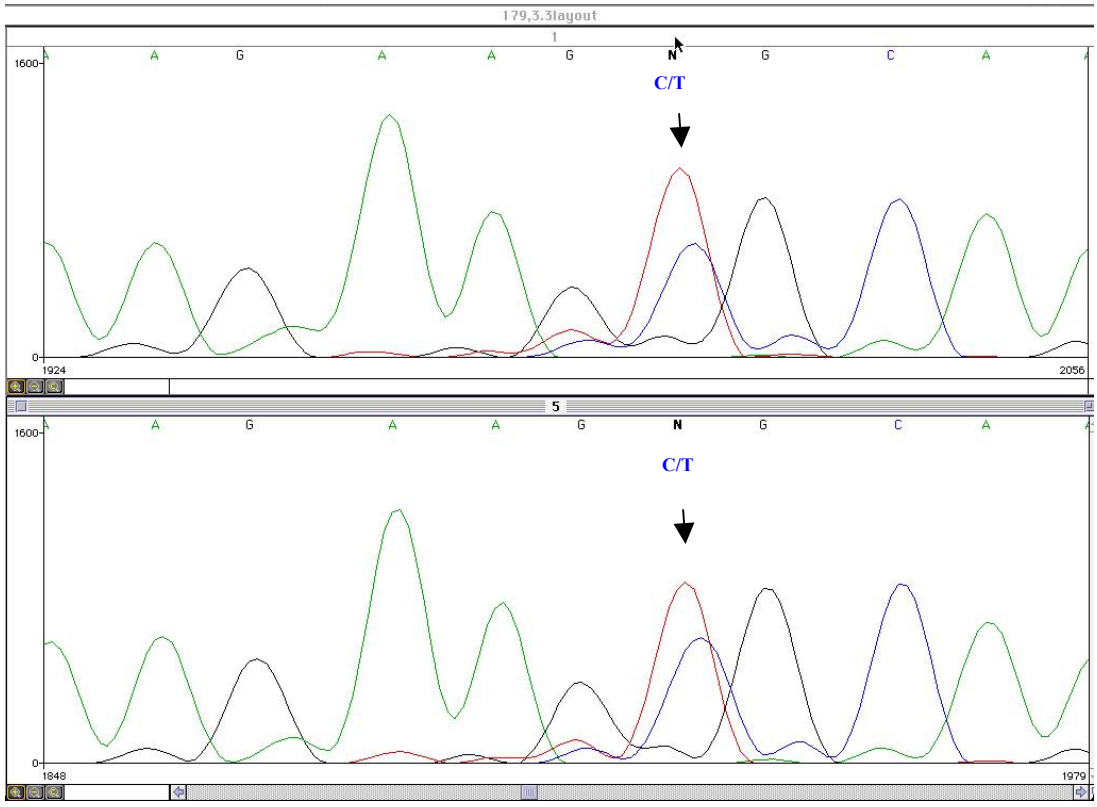


Figure 35. Electropherogram showing R306C (916 C to T) mutation.

a. Sample 00-179: DNA sequence obtained with forward primer,
b. Sample 00-179: DNA sequence obtained with reverse primer.

3.7.5. *MECP2* exon 4.3 R270X mutation

Cycle sequencing reactions were performed using *MECP2* exon 4.3 primers, GA 534 and GA 535 and ABI prism kit, in order distinguish the mutation type, whether it was R270X or V288X. The samples which were found to had mutation by RE digestion, 99-104, 00-187 and 00-196 were sequenced. By using ABI Sequence Analysis Software on the 377 DNA Sequencer, the DNA products were electrophoresed and analyzed. Heterozygous bases were identified using the Factura software program. Then, finally the DNA sequence was aligned with the reference sequence using the Sequence Navigator Software Program. The only difference between the reference sequence and 99-104, 00-187 and 00-196 sample was at nucleotide 916 in exon 4.3. At this position, although there was C (cytosine) residue in the reference sequence, the samples were heterozygous "N" with one allele having a C (cytosine) residue and the other allele having a T (tyrosine) residue at this point (figure 36). The sequence result of 00-196 will be explained and given in "somatic mosaicism" section.

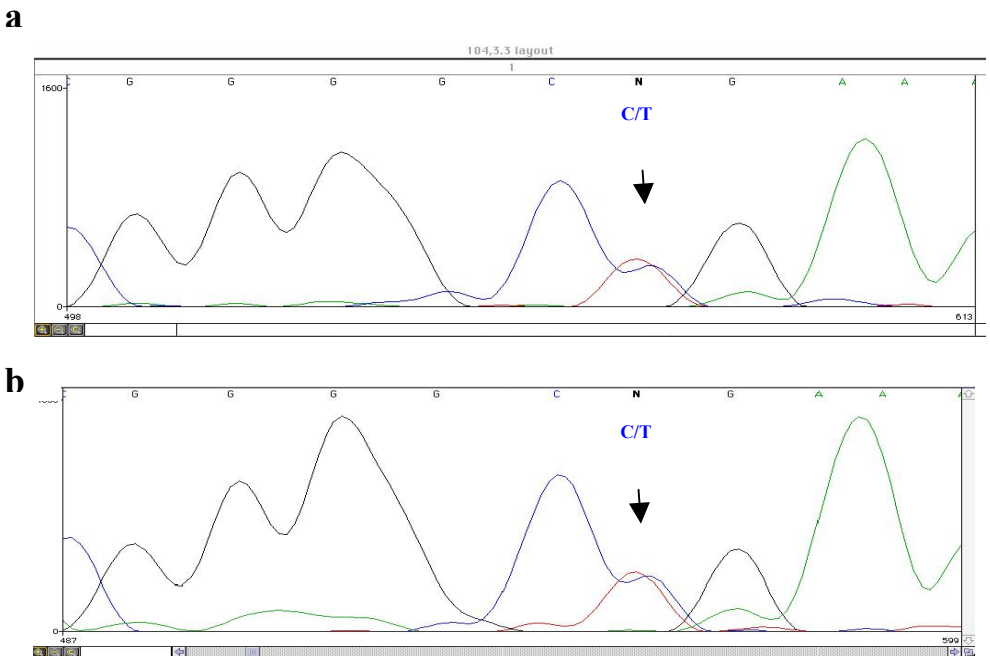


Figure 36. Electropherogram showing R270X (808 C to T) mutation.

a. Sample 99-104: DNA sequence obtained with forward primer,
b. Sample 00-187: DNA sequence obtained with forward primer.

3.7.6. *MECP2* exon 4.1 T197M mutation

Cycle sequencing reactions were performed using *MECP2* exon 4.1 primers, GA 530 and GA 531 and ABI prism kit, for 99-114 sample in order to identify the different pattern, that leaded to mutation, after *Nla*III digestion. By using ABI Sequence Analysis Software on the 377 DNA Sequencer, the DNA products were electrophoresed and analyzed. Heterozygous bases were identified using the Factura software program. Then, finally the DNA sequence was aligned with the reference sequence using the Sequence Navigator Software Program. The only difference between the reference sequence and 99-114 sample was at nucleotide 590 in exon 4.1. At this position, although there was C (cytosine) residue in the reference sequence, the 99-114 sample was heterozygous "N" with one allele having a C (cytosine) residue and the other allele having a T (tyrosine) residue at this point (figure 37). Thus, after restriction enzyme digestion results, it was identified a novel mutation; T197M; in sample 99-114 by sequencing.

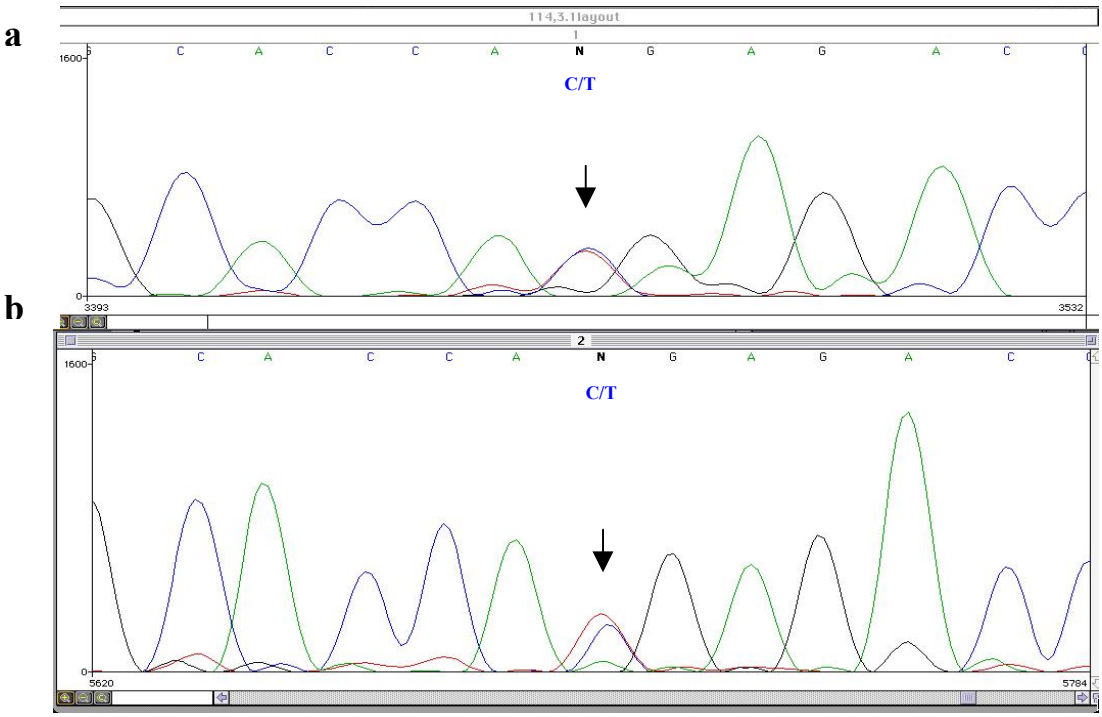


Figure 37. Electropherogram showing T197M (590 C to T) mutation.

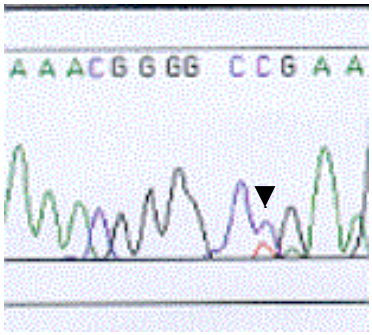
a. Sample 00-132: DNA sequence obtained with forward primer,
b. Sample 00-132: DNA sequence obtained with reverse primer.

3.8. Somatic mosaicism for R270X mutation in a boy with classical RTT

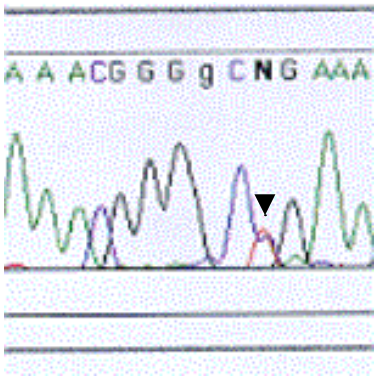
A R270X mutation was identified in a boy, 00-196, with RTT. Also this mutation was detected in two girls with RTT (99-104, 00-187) (figure 38 B). The mutant allele in the affected boy was found along with the wild-type allele through reduced dosage when compared with the samples of the females. Although, the mutant (T) and wild-type (C) nucleotide peaks were equal in the girls, the mutant (T) nucleotide peak of 00-196 was lower than the wild-type nucleotide (C) peak. (figure 38A a,b). In order to perform dosage analysis of the alleles, genomic DNA of 00-196 and 99-104 was re-amplified with the same primers used in sequencing reaction, and digested again with *Nla*IV restriction enzyme. The restriction fragment's densitometric scanning revealed that the ratio is 56:44 in the girl and 36:64 in the boy (figure 38 B).

A

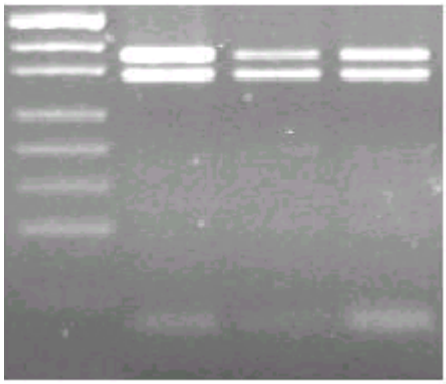
a. 00-196 (male RTT)



b. 99-104 (female RTT)



B



%T allele	56 (50-64)	41 (36-47)	36 (28-43)
%C allele	44 (36-50)	59 (53-64)	64 (57-72)

Figure 38. 00-196 sample which is somatic mosaic for R270X.

A. Sequence analysis of samples 00-196 and 99-104,
B. Restriction digestion result of female 99-104 blood, male 00-196 hair root and 00-196 blood

Also restriction results from 00-196's PCR products from hair sample, indicated similar ratio (figure 38 B). Cytogenetic analysis showed that 00-196 has normal 46,XY karyotype. All these findings suggest that 00-196 is a somatic mosaic for R270X.

3.9. X-inactivation assay

The androgen receptor (AR) assay was used to assess X chromosome inactivation pattern in RTT families (Allen et al. 1992). This assay is based on the differential methylation of a cytosine residue just 5' to the highly polymorphic trinucleotide repeat in the androgen-receptor gene. On inactive X this site is methylated so that, is resistant to digestion by methylation-sensitive *Hpa*II enzyme. Conversely, the site is unmethylated on active X and susceptible to digestion. *Hpa*II-digested genomic DNA is used as a template for PCR reaction. This amplification product allows determination of the relative ratio of methylation of each allele, corresponding to the ratio of inactivation. The polymorphic triplet repeat, adjacent to the methylation site, provides a length difference between the alleles. This difference serves to separate the two alleles. The X chromosome inactivation pattern is considered skewed when the same X chromosome appears to be inactive in ≥80 % of cells (Naumova et al. 1996).

The androgen-receptor assay was performed on genomic DNA from the female RTT patients who has mutation of *MECP2*. Figure 39 represents three of the sample's, 00-133, 00-188, and 00-196 (male patient) AR assay results. Sample from the male RTT patient with mutation is amplified only if undigested by *Hpa*II, which is consistent with the presence of a single active AR gene. In all samples, except sample 00-188 random X chromosome inactivation is found. Table 9 summarizes the result of X inactivation study.

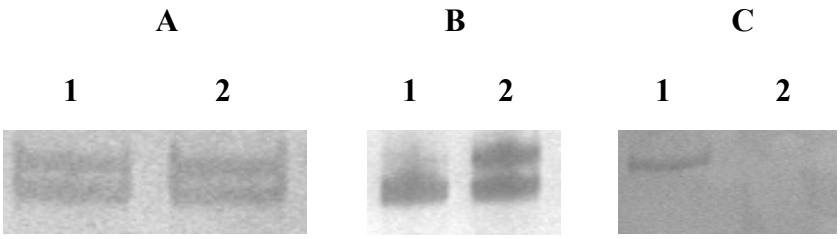


Figure 39. X chromosome inactivation pattern of 00-133, 00-188, 00-196.

A. Lane 1: *Hpa*II-digested PCR product of 00-133; lane 2: Undigested PCR product of 00-133.
B. Lane 1: *Hpa*II-digested PCR product of 00-188; lane 2: Undigested PCR product of 00-188.
C. Lane 1: Undigested PCR product of 00-196; lane 2: *Hpa*II-digested PCR product of 00-196.

Table 9 X inactivation patterns of RTT patients with *MECP2* mutation

No	Family Code	Pt Code	Domain	Exon	Base Change	AA change	Mutation designation	X-inactivation (AR)
1	RTT-4	99-91	MBD	3	316 C to T	Arg to Trp	R106W	random
2	RTT-34	00-163	MBD	3	316 C to T	Arg to Trp	R106W	Not informative
3	RTT-42	00-174	MBD	3	316 C to T	Arg to Trp	R106W	random
4	RTT-19	00-133	MBD	4	455 C to G	Pro to Arg	P152R	random
5	RTT-28	00-157	MBD	4	455 C to G	Pro to Arg	P152R	Not informative
6	RTT-18	00-132	MBD	4	473 C to T	Thr to Met	T158M	random
7	RTT-51	00-188	MBD	4	473 C to T	Thr to Met	T158M	Skewed
8	RTT-60	00-201	MBD	4	473 C to T	Thr to Met	T158M	random
9	RTT-26	00-155	MBD	4	473 C to T	Thr to Met	T158M	random
10	RTT-9	99-107	MBD	4	473 C to T	Thr to Met	T158M	random
11	RTT-50	00-187	TRD	4	808 C to T	-	R270X	random
12	RTT-8	99-104	TRD	4	808 C to T	-	R270X	random
13	RTT-14	00-43	TRD	4	916 C to T	Arg to Cys	R306C	random
14	RTT-15	00-44	TRD	4	916 C to T	Arg to Cys	R306C	Not informative
15	RTT-6	99-95	TRD	4	916 C to T	Arg to Cys	R306C	random
16	RTT-44	00-179	TRD	4	916 C to T	Arg to Cys	R306C	random
17	RTT-31	00-160	TRD	4	916 C to T	Arg to Cys	R306C	random
18	RTT-16	00-45		4	1163 del44nt	-	del	random
19	RTT-48	00-184		4	1163 del44nt	-	del	random

3.10.Genotype-Phenotype correlations

In order to compare the clinical features with the mutational spectrum in *MECP2*, a detailed clinical scoring system was used. The RTT patients harbouring different mutations were assessed for fourteen clinical symptoms. Three patients were with forme fruste variant and sixteen with classical RTT. Except one patient the others can not use their hands. The other symptoms are highly variable (table 10 and 11).

The patients who harbour same mutation do not display similar symptoms. As a result, there is not any consistent correlation between clinical severity and the type of mutation.

Table 10 Genotype-phenotype correlation

Pt Code	*Mutation design.*	*Type of RTT*	*Onset (mo)*	*Ambulation*	*Seizures*	*Respiratory dysfunction*	*Head growth*	*Somatic growth failure*	*Motor*	*Hand use*	*Communication*	*Autonomic dysfunction*	*EEG*	*Scoliosis*	*Self-abuse*	*Screaming*
99-91	R106W	cl	3	0	0	1	3	1	0	3	1	0	NA	0	0	0
00-163	R106W	FF	3	0	0	0	3	0	1	3	1	1	1	0	0	2
00-174	R106W	FF	4	3	2	0	0	0	2	3	2	0	1	0	0	0
00-133	P152R	cl	3	3	1	1	2	0	2	3	1	1	1	0	2	2
00-157	P152R	cl	1	0	1	0	3	0	0	3	0	0	2	0	1	1
00-132	T158M	cl	2	0	0	0	0	0	1	3	0	0	2	1	1	2
00-188	T158M	FF	5	3	0	0	0	0	2	3	0	0	2	0	0	2
00-201	T158M	cl	3	0	0	0	2	2	0	3	1	1	1	0	1	0
00-155	T158M	cl	4	3	2	0	3	?	2	3	1	1	1	0	2	1
99-107	T158M	cl	2	1	2	2	3	2	0	3	?	1	1	0	0	0
00-187	R270X	cl	5	3	2	2	2	2	0	3	?	1	?	0	0	0
99-104	R270X	cl	2	1	1	0	3	1	1	2	1	0	1	1	0	2
00-43	R306C	cl	1	1	0	0	2	0	0	3	1	0	2	0	0	0
00-44	R306C	cl	2	1	2	2	3	?	1	3	1	1	?	1	?	1
99-95	R306C	cl	1	2	2	3	3	2	0	3	1	1	?	0	?	1
00-179	R306C	cl	1	0	1	0	3	0	0	3	?	0	2	0	?	0
00-160	R306C	cl	1	1	1	1	3	0	1	3	1	0	2	0	?	0
00-45	del	cl	2	1	1	0	3	?	0	3	1	?	2	1	?	1
00-184	del	cl	2	0	0	0	3	1	1	3	?	?	1	0	1	1

Table 11 Severity score for RTT

Variable	Sc.	Definition	Variable	Sc.	Definition
Age at onset	1	> 30 mo	Hand use	0	feeds self
	2	18-20 mo		1	holds objects
	3	12-18 mo		2	may reach for objects
	4	6-12 mo		3	none
	5	< 6 mo			
Ambulation	0	walks alone	Communication	0	makes choices
	1	walks with help		1	inconsistent eye gaze
	2	used to walk		2	no communication
	3	never walked			
Seizures	0	None	Autonomic dysfunction	0	none
	1	well controlled		1	mild color/temperature changes
	2	uncontrolled		2	moderate
	3	infantile spasms		3	severe
Respiratory dysfunction	0	None	Electroencephalogram	0	normal pattern
	1	minimal cyanosis		1	multifocal and/or slow spike
	2	intermittent cyanosis		2	slow spike; slow background
	3	constant cyanosis		3	hypsarrhythmia
Head growth	0	none to minimal deceleration	Scoliosis	0	none
	1	deceleration > 10th percentile		1	minimal: $<20^0$
	2	2nd-10th percentile after 24 mo		2	moderate: $<20^0$-70
	3	2nd percentile by 24 mo		3	severe $>70^0$
	4	< 2nd percentile by 24 mo			
Somatic growth	0	no growth failure	Self abuse	0	none
	1	mild failure		1	intermittent
	2	moderate failure requiring		2	constant
		oral supplements			
Motor	0	none to mild ↑ or ↓ tone	Screaming	0	none
	1	moderate tone change and mild		1	< weekly
		↓ ambulation		2	weekly or more
	2	severe impairement of ambulation			

4. Discussion

With the discovery of *MECP2* mutations, Rett Syndrome became the first human disease found to be caused by defects in a protein involved in the regulation of gene expression through its interaction with methylated DNA (Amir et al., 1999, Wan et al., 1999, Ng et al., 1999a, Li et al., 1992, Tate et al., 1996).

4.1. Mutation Analysis

In this study 63 RTT patients classified as classical RTT (n=43), RTT variant (n=14), male RTT (n=4), and familial RTT (n=2) were screened for seven recurrent mutations (R106W, P152R, T158M, R306C, R168X, R270X, V288X), one rare mutation (F155S), and one polymorphism (E397K). Restriction enzyme analysis was used to analyze these known mutations. DNA sequencing was performed to confirm the results (table 12).

In the classical RTT group R106W was found in one (2.32%) patient, P152R in two (4.65%) patients, T158M in four (9.3%) patients, R306C in five (11.6%) patients, R270X in two (4.65%) patients, del 44 nt in two (4.65%) patients. E397K polymorphism was detected in one patient from this group.

In the RTT variant group R106W was found in two (14.28%) patients, T158M in one (7.14%) patient, and the novel T197M mutation in one (%7.14%) patient.

Among the four male RTT patients one (25%) was found to carry the R270X mutation along the normal MECP2 sequence. Finally, no mutation was detected among the familial cases. R168X and V288X mutations were found in any one of the groups either.

Table 12 Distribution of the mutations in the patient groups

	RTT-classic (n=43)	RTT-variant (n=14)	RTT-male (n=4)	RTT-familial (n=2)
Mutation				
R106W	1 (2.32%)	2 (14.28%)	0	0
P152R	2 (4.65%)	0	0	0
F155S	0	0	0	0
T158M	4 (9.3%)	1 (7.14%)	0	0
R168X	0	0	0	0
T197M	0	1 (7.14%)	0	0
R270X	2 (4.65%)	0	1 (25%)	0
V288X	0	0	0	0
R306C	5 (11.6%)	0	0	0
del 44 nt	2 (4.65%)	0	0	0
Total	16 (37.2%)	4 (28.57%)	1 (25%)	0

Although, R168X mutation has been reported as the most frequent mutation with frequency ranging from 12 to 39 % of all mutations found in the studies (Wan et al., 1999, Amir et al., 2000a), we did not see this mutation in our patients. Thus, R168X mutation is not considered as a common mutation in our Turkish RTT patients. R168X mutation frequency might change from one population to another since it was found rarely also in the Swedish RTT patients (Erlandson et al., 2001).

One novel mutation, T197M, was identified in one of our patients. It was known that because of the hypermutability of CpG sites, C to T transitions form the majority of the *MECP2* mutations. Consistent with this observation, the novel mutation that we identified is a C to T transition. This C590T transition is found in a

region which is important for interaction of *MECP2* with Sin3A which is a co-repressor in the histone deacetylase complex (Nan et al., 1998b). Thus this mutation most probably affects the interaction of MeCP2 with co-repressor and disrupts the MeCP2- repressor complex integrity.

While performing the mutation screening experiments, a boy who is mosaic for the recurrent R270X was identified. After a boy who was reported as having somatic mosaicism in the *MECP2* gene, our case is the second RTT patient who is mosaic for a *MECP2* mutation (Clayton-Smith et al., 2000). In the previous report the patient was mosaic for a rare *MECP2* mutation. However, in our case, he has somatic mosaicism for a recurrent *MECP2* mutation.

Somatic mosaicism has been reported in different genetic diseases including X-linked disorders; ornithine transcarbamylase deficiency (Maddalena et al., 1988), double cortex syndrome/X-linked lissencephaly (Gleeson et al., 2000), Duchenne muscular dystrophy (Bakker et al., 1989), and hemophilia A (Gitschier et al., 1989, Oldenburg et al., 2000). Thus, somatic mosaicism should be considered when there is a male patient with an X-linked dominant disease.

Our *MECP2* mutation detection rate is 37.2% for the classical group, 28.57% for the variant group, and 25% for the male group. This rate can be increased by screening for other recurrent mutations such as R255X, and performing DNA sequencing for the entire MECP2 gene. Since this was not cost-effective, it was not performed at this stage in our laboratory.

4.2. X-chromosome inactivation (XCI)

To determine whether patterns of X chromosome inactivation contributes to phenotypic variability, 19 RTT patients with *MECP2* mutations were evaluated. 16 patients who were analysed via AR assay were informative. In these patients 15 has random and 1 has skewed X inactivation in blood. Also, it was seen that the patient with skewed X-inactivation does not represent a milder phenotype. This may be due to somatic mosaicism for the X-inactivation patterns in different tissues.

As a consequence, the X chromosome inactivation pattern can not be related to the phenotype which is consistent with the previous study (Nielsen et al., 2001). The random XCI pattern in most of the RTT patients suggests that the RTT phenotype is seen because of the high expression of mutant *MECP2* in cells.

4.3. Genotype- Phenotype Correlation

In reviewing the phenotypes reported for a mutation, a wide variability in each of the domains were examined (Amir et al., 1999, Wan et al., 1999, Amir et al., 2000a, Cheadle et al., 2000, Xiang et al., 2000, Bienvenu et al., 2000, Huppke et al., 2000). Positive correlation between mutations and phenotype was reported only in Amir et al. 2000a. A correlation was found between truncating mutations and two parameters: breathing abnormalities and low levels of CSF HVA (a breathing dysrhythmia, with periods of hyperventilation or apnea, breath-holding, interrupting normal breathing). Also they showed that patients with missense mutations were more likely to have scoliosis than with truncating mutations (Amir et al., 2000a).

We did not detect any correlation between genotype and phenotype (table 10). The correlation that was shown between truncating mutation and breathing dysfunction and missense mutation and scoliosis was not found in our RTT patients with mutations. Except for one patient, the other patients can not use their hands. The other characteristics, including major clinical features are different from one patient to another.

5. Future Perspectives

Since only seven recurrent mutations were screened until now, additional mutation analysis studies should be performed. As a first step, R255X recurrent mutation can be screened via DNA sequence analysis, since this mutation does not make a change at the cleavage site of any enzyme. Second, our RTT patients should be screened for mutations via DNA sequencing analysis for the entire coding region of the *MECP2* gene. Third, *MECP2* introns can be analysed to see if structure or function of the gene is altered by intronic mutations.

One of our male patients with classical RTT was found to be mosaic for the R270X mutation. Single cell clones of this patient from blood cells can be obtained to analyse the global expression pattern of genes to better understand the underlying pathology in RTT.

After completing the whole *MECP2* gene mutation screening, a more comprehensive genotype-phenotype correlation can be performed and a consistent correlation between the symptoms and the mutations might be established.

As MeCP2 protein is involved in regulation of gene expression, it is likely that mutations of *MECP2* will cause misregulation of downstream genes, which are normally silent. This is probably the underlying pathology of clinical features in RTT. There are a large numbers of genes on the X chromosome, which can be candidate downstream genes that are effected by *MECP2* mutations. These candidate genes can be analysed in cell lines derived from a single cell clone of RTT patients to understand whether there is inappropriate expression of the genes. The result of this study might demonstrate if gene expression profile on the inactive X chromosome is altered in RTT or not.

The other mechanism that is effected by methylation-dependent gene silencing is genomic imprinting. In order to analyze the effects of *MECP2* mutations on the imprinting process genes subject to genomic imprinting can be analysed in RTT patients. While methylation of imprinted allele is lost and imprinted allele is reactivated in cells with mutant *MECP2*, biallelic rather than monoallelic expression of imprinted genes would be recognized.

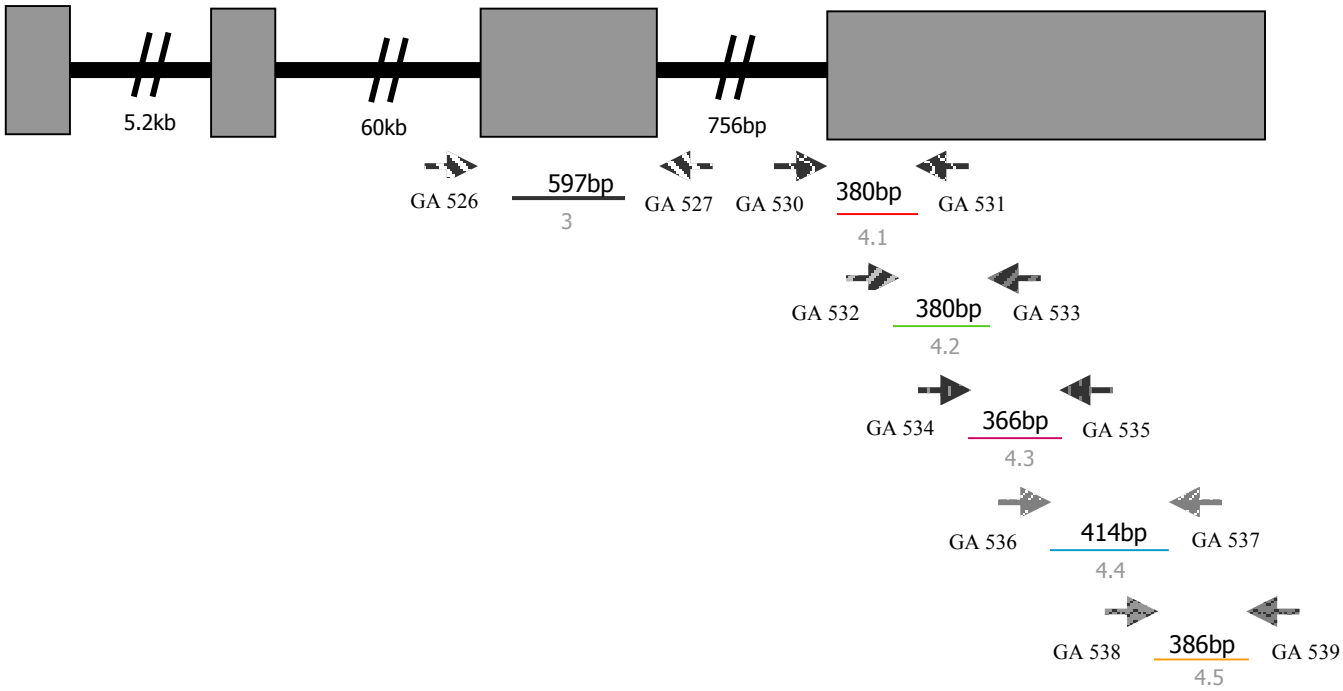
The identification of mutations in *MECP2* gene as the cause of RTT provides a good opportunity to study the pathogenesis of the disease at the molecular level. As the mouse *MECP2* gene is highly similar to the human gene, studies of the mouse gene may help us to establish an animal model for RTT. Using the conditional knockout technology, mice that lack *MECP2* either in all tissues or selectively in the brain was generated (Bird et al., 1999, Guy et al., 2001). The mutant mice show features that are similar to those in RTT, including apparent normal pre- and perinatal development but fast postnatal deterioration. These *MECP2* mutant

mice can be analyzed, focusing on revealing any cellular defects associated with MeCP2 deficiency in the mouse central nervous system. Since there is behavioral defects in RTT, it will be interesting to analyze whether there is also behaviral defects in mice. The result of this study might help to cure the disease because of the identification of RTT pathogenesis.

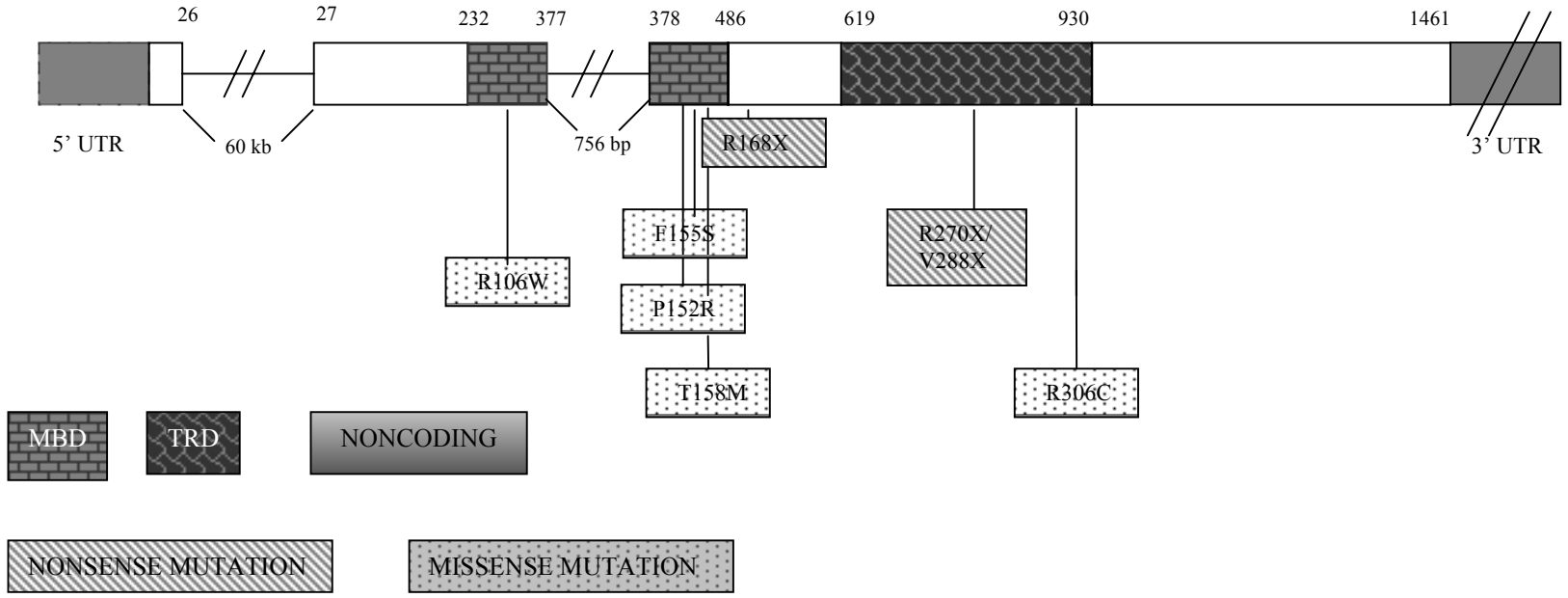
After identifying the function of MeCP2 totally, pathogenesis of RTT and the downstream gene products, a cure for RTT may be in reach. Drugs, gene therapy and may be stem cell, with normal *MECP2*, transplantation can be the solution for the treatment of this syndrome.

APPENDICES

Appendix 1. Schematic representation of the PCR product sizes and primers that were used to amplify the *MECP2* gene.



Appendix 2. The mutations that were screened in *MECP2* gene.



Appendix 3.

Mutations	Location	Domain	Nucleotide change[a]	Aminoacid change	Restriction site[b]
Missense	Exon 2	MBD	C291A	D97E	None
	Exon 2	MBD	C301A	P101T	None
	Exon 2	MBD	C302A	P101H	Nla III(+)
	Exon 2	MBD	C302A	P101L	None
	Exon 2	MBD	G317A	R106Q	None
	Exon 2	MBD	C316T	R106W	Nla III(+)
	Exon 3	MBD	C397T	R133C	None
	Exon 3	MBD	G398T	R133L	None
	Exon 3	MBD	C401G	S134C	None
	Exon 3	MBD	C455G	P152R	Nla IV (-)
	Exon 3	MBD	T463A	F155I	None
	Exon 3	MBD	T464C	F155S	Tfi(+)
	Exon 3	MBD	C468G	D156E	None
	Exon 3	MBD	C473T	T158M	Nla III(+)
	Exon 3	MBD	G481T	G161 W	Bsr I(-)
	Exon 3	TRD	C674G	P225R	None
	Exon 3	TRD	C904G	P302A	None
	Exon 3	TRD	C905G	P302R	Bse RI(+)
	Exon 3	TRD	C905T	P302L	None
	Exon 3	TRD	C916T	R306C	Hha I(-)
	Exon 3	TRD	G917A	R306H	Hha I(-)
	Exon 3	TRD	C964G	P322A	Dra II(+)
	Exon 3	TRD	C965T	P322L	Alu I(+)
	Exon 3	CTS	A1461C	X487C	Fnu4HI (+)
Nonsense	Exon 2		C129T	Q19X	Bpm I
	Exon 2		C146A	S49X	None
	Exon 3	MDB	C423G	Y141X	None
	Exon 3	CRIR	C502T	R168X	Hph I(+)
	Exon 3	CRIR	C508T	Q170X	None
	Exon 3	CRIR	A592T	R198X	Mae II(+)
	Exon 3	TRD	C763T	R255X	None
	Exon 3	TRD	A766T	K256X	None
	Exon 3	TRD	C808T	R270X	Nla IV(-)
	Exon 3	TRD	C880T	R294X	None
Splicing site	Intron 2	MBD	A378-2C	L138X	Bsl I(+)
	Intron 2	MBD	A378-2G		Nla IV (+)
Deletions/ insertions	Exon 3	MBD	258delCA		None
	Exon 3	MBD	407del507+ins8		
	Exon 3	MBD	411delG	L138X	None
	Exon 3	MBD	431delA		None
	Exon 3	MBD	439delG	V159X	None
	Exon 3	TRD	654del4bp		None
	Exon 3	TRD	706delG		None
	Exon 3	TRD	730del436bp	P391X	
	Exon 3	TRD	803delG		None
	Exon 3	TRD	806delG	V288X	Nla IV(-)
	Exon 3	CRIR	620insT	E235X	Bsg I(-)
	Exon 3	TRD	677insA		None
	Exon 3	TRD	747dup5bp	V288X	Fau I(+)
	Exon 3	CTS	1127ins10bp		
	Exon 3	CTS	1194insT		Bspw I(+)
	Exon 3	CTS	1195insT		None

Mutations	Location	Domain	Nucleotide change[a]	Aminoacid change	Restriction site[b]
	Exon 3	CTS	1364insC		None
	Exon 3	CTS	1150del43bp	P403X	
	Exon 3	CTS	1157del31bp	P403X	
	Exon 3	CTS	1158del43bp	P403X	
	Exon 3	CTS	1164del43bp	P403X	
	Exon 3	CTS	1096-1165del 7 to 170bp		

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