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Diagnostic mutational analysis of *MECP2* in Korean patients with Rett syndrome

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Abbreviations: HDAC, histone deacetylases; MBD, methyl-CpG binding domain; MECP2, methyl-CpG binding protein 2; NLS, nuclear localization signal; RTT, Rett syndrome; SNP, single nucleotide polymorphism; TRD, transcriptional repression domain; WDR, group II WW domain binding region

Abstract

Rett syndrome (RTT) is an X-linked dominant neurodevelopmental disorder affecting 1 per 10,000-15,000 female births worldwide. The disease-causing gene has been identified as *MECP2* (methyl-CpG-binding protein 2). In this study, we performed diagnostic mutational analysis of the *MECP2* gene in RTT patients. Four exons and a putative promoter of the *MECP2* gene were analyzed from the peripheral blood of 43 Korean patients with Rett syndrome by PCR-RFLP and direct sequencing. Mutations were detected in the *MECP2* gene in approximately 60.5% of patients (26 cases/43 cases). The mutations consisted of 14 different types, including 9 missense mutations, 4 nonsense mutations and 1 frameshift mutation. Of these, three mutations (G161E, T311M, *p385fsX409*) were newly identified and were determined to be disease-causing mutations by PCR-RFLP and direct sequencing analysis. Most of the mutations were located within MBD (42.3%) and TRD (50%). T158M, R270X, and R306C mutations were identified at a high frequency. Additionally, an intronic SNP (IVS3 + 23C > G) was newly identified in three of the patients. IVS3 + 23C > G may be a disease-related and Korea-specific SNP for RTT. L100V and A201V are apparently disease-causing mutations in Korean RTT, contrary to previous studies. Disease-causing mutations and polymorphisms are important tools for diagnosing RTT in Koreans. The experimental procedures used in this study should be considered for clinical molecular biologic diagnosis.

Keywords: DNA mutational analysis; diagnosis; MECP2 protein, human; polymorphism, restriction fragment length; Rett syndrome

Introduction

Rett syndrome (RTT, MIM No. 312750) is an X-linked dominant neurodevelopmental disorder and is the second most common cause of mental retardation in females, following Down syndrome (Rett, 1966; dos Santos *et al*., 2005). The vast majority of cases of RTT (more than 99%) are sporadic occurrences and familial recurrences are rare (Hoffbuhr *et al*., 2001).

RTT is caused by mutations in a gene encoding the methyl-CpG binding protein 2 (*MECP2*, AF30876) (Amir *et al*., 1999). *MECP2* is mapped between *IRAK* (interleukin-1 receptor associated kinase) and *RCP* (red opsin gene) loci on chromosome Xq28 (Reichwald *et al*., 2000). It is 76 kb in size and is

composed of 486 amino acids. The four exons of human *MECP2* have a combined length of 1775 bp. *MECP2* participates in transcriptional silencing by binding to methylated DNA in nucleosomes and chromatin. It contains functional domains, a methyl-CpG binding domain (MBD) of 85 amino acids that binds to methylated CpG islands, and a transcriptional repression domain (TRD) of 104 amino acids that interacts with the transcriptional repressor Sin3A, which recruits histone deacetylases (HDAC) (Van den Veyver and Zoghbi, 2000). In addition, *MECP2* has a nuclear localization signal (NLS) (Jorgensen and Bird, 2002) and a group II WW domain binding region (WDR) (Buschdorf and Stratling, 2004; Weaving *et al*., 2005). The function of NLS within the TRD location is to facilitate the transport of *MECP2* into the nucleus.

There are only a few reports regarding mutational analysis of *MECP2* in Koreans because the overall understanding of RTT is limited (Chae *et al*., 2002). To compare the pattern of *MECP2* mutations in Koreans with RTT from worldwide reports, we performed mutational analysis in Korean RTT patients and control subjects. Direct DNA sequencing was used and results were confirmed by PCR-RFLP.

Materials and Methods

DNA samples from RTT patients

Whole blood samples (500 μl) from 43 sporadic RTT patients (41 females and 2 males) were collected in EDTA tubes. All patients were phenotypically classical RTT. Genomic DNA was extracted using an E.Z.N.A. blood DNA kit (Omega Bioteck Inc. Norcross).

PCR amplification

The putative promoter and four exons of the *MECP2* gene were amplified by PCR by dividing exon 2 into two parts, exon 3 into two parts, and exon 4 into five parts. Primer sequences designed by Amir *et al*. (1999) were used, except for the putative promoter and exon 1. The sequence of the promoter and exon 1 were 5'-gggTgCAATgAAACgCTTA-3' (forward) and 5'-TTTACCACAgCCCTCTCTCC-3' (reverse).

Direct sequencing

The PCR products were cleaned using a QIAquick gel extraction kit (QIAGEN, Hiden, Germany). The sequencing primers were the same as those for PCR. The DNA sequencing results were compared with the normal DNA sequence (AF030876).

RFLP analysis

We used the GeneTyx program to investigate the restriction enzyme sites. *Dde* I (New England Biolabs Co., MA) was used to detect G161E. *BsmB* I (New England Biolabs.) was used to detect T311M. A mismatch PCR technique was used to determine if the L100V is a disease-causing mutation. A second new reverse primer of exon 3 for the L100V mutation was designed (5'-GCTTAAGCTTCCGTGTCCAGCCTTCAGGTA-3'). The primer sequence for mismatch PCR is underlined. The PCR product for

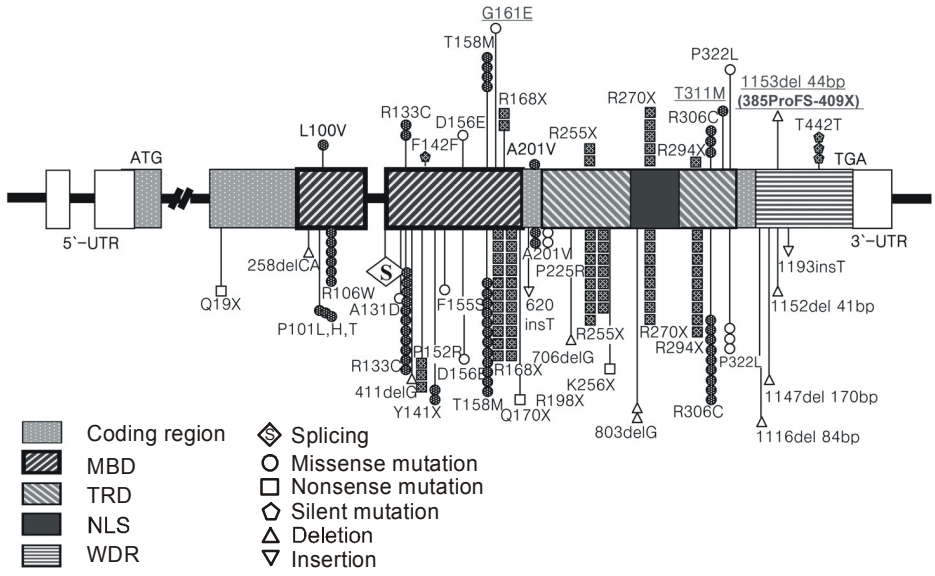

Figure 1. Distribution of *MECP2* mutations in RTT. Top: Mutations identified in this study. Novel mutations are indicated by bold underlined type. Bottom: mutations described previously (*MECP2* variation database of InterRETT and RettBASE) (Fyfe *et al*., 2003). Circles (○, ●) represent missense mutations and squares (□, ■) represent nonsense mutations. Mutations at CpG dinucleotides are shown by filled circles (●) or squares (■). Frequency is indicated by the number of symbols (Buyse *et al*., 2000; Miltenberger-Miltenyi and Laccone, 2003; Fukuda *et al*., 2005; Oexle *et al*., 2005).

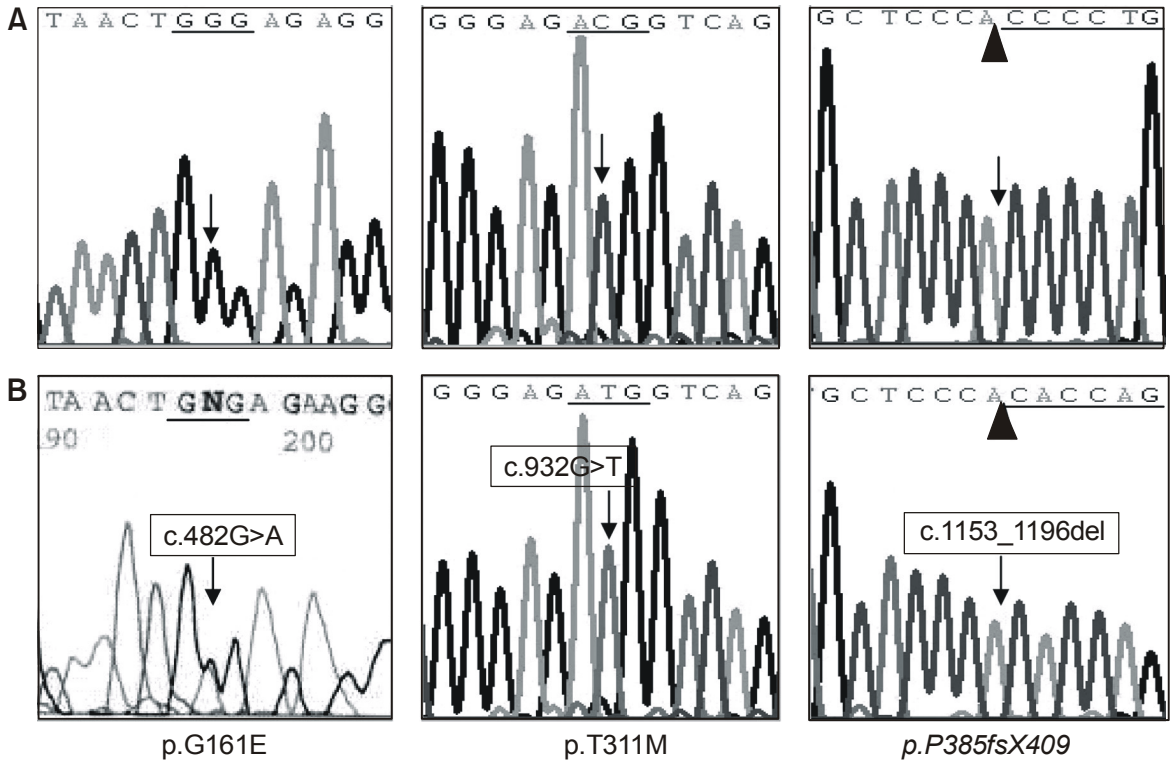

Figure 2. DNA sequencing electropherograms of wild type (A) and novel mutations (B) of *MECP2* identified in RTT patients in this study. Substituted nucleotides are indicated by arrows and substituted amino acids are underlined. Deletion sites are indicated by arrowheads. All sequences are in the sense orientation. The mutation *P385fsX409* was identified by sequencing after cloning.

L100V in the *MECP2* gene created an *Afa* I (Takara, Tokyo, Japan) restriction site. A201V created a *Bal* I (Takara) restriction site. Restriction digested products were separated by electrophoresis on either 2% agarose gel or 4% NuSieve gel (FMC, Rockland) (Bedia *et al*., 2003; Kim *et al*., 2004).

Results

Mutational analysis of *MECP2*

To investigate mutation of the *MECP2* gene, we analyzed a promoter and four exons of *MECP2* in 43 RTT patients by direct sequencing. The amplified PCR product contained an intron and an exon. *MECP2* mutations were detected in 26 (60.5%) of the 43 patients. These results were compared with the *MECP2* variation database for InterRETT and RettBASE (Fyfe *et al*., 2003). The mutations consisted of 14 different types, including 9 missense mutations, 4 nonsense mutations, and 1 frameshift mutation. Most of these mutations were located within MBD (42.3%) and TRD (50%) (Figure 1). Of the mutations identified, three (G161E, T311M, *P385fsX409*) were new (Figure 2). Three mutations, T158M (9.3%), R270X (11.6%), and R306C (7%), were identified with a high frequency (Table 1). The two male patients with different RTT phenotypes did not have any identifiable mutations. Two silent mutations (F142F, T442T) that had been reported previously (Miltenberger-Miltenyi and Laccone, 2003) were also found. These accompanied other mutations in three of the four patients with F142F and T442T.

Confirmation of the mutations

To confirm the novel sequence changes, RFLP analysis was performed for G161E and T311A (Table 1) and direct sequencing was performed for *p385fsX409*. The G161E (c.482G > A) mutation created a *Dde* I site while the wild type had 241 bp and 129 bp fragments, and the heterozygotic mutant had 241 bp, 202 bp, 129 bp, and 39 bp fragments (Figure 3A, lane 5 and 6). The T311A (c.932C > T) mutation destroyed the *BsmB* I site. The 366 bp product was digested into 198 bp and 168 bp fragments in the wild type (Figure 3A, lane 8), whereas patients with T311A exhibited a single band

Table 1. Summary of *MECP2* mutations and sequence variants in RTT patients

Mutation type	Domain	Exon	Nucleotide change[a]	Amino acid change[a]	Frequency[b]	Restriction enzyme[e]	Patients
Missense mutations	MBD	Exon3(2)	c.298C > G	p.L100V	2.3%	*Afa* I(+)	R21
	MBD	Exon4(1)	c.397C > T	p.R133C	4.7%		R8, 35
	MBD	Exon4(1)	c.468C > G	p.D156E	2.3%		**R24**
	MBD	Exon4(1)	c.473C > T	p.T158M	9.3%		R6, 11, 12, 19
	MBD	Exon4(1)	c.482G > A	p.G161E[c]	2.3%	*Dde* I(+)	R13
	IDR	Exon4(1)	c.602C > T	p.A201V	2.3%	*Bal* I(+)	R20
	TRD	Exon4(3)	c.916C > T	p.R306C	7.0%		**R4**, 30, 33
	TRD	Exon4(3)	c.932C > T	p.T311M[c]	2.3%	*BsmB* I(-)	R28
	WDR, C-term.	Exon4(3)	c.965C > T	p.P322L	2.3%		R16
Nonsense mutations	MBD	Exon4(1)	c.502C > T	p.R168X	4.7%		R5, **7**
	TRD	Exon4(2)	c.763C > T	p.R255X	4.7%		R9, 14
	TRD, NLS	Exon4(3)	c.808C > T	p.R270X	11.6%		**R15**, 23, **25**, 31, 38
	TRD	Exon4(3)	c.880C > T	p.R294X	2.3%		R36
Frameshift	WDR, C-term.	Exon4(4)	c.1153_1196del	*p.P385fsX409*[c]	2.3%		R37
Silent mutations	MBD	Exon4(1)	c.426C > T	p.F142F[d]	2.3%		**R4**
	WDR, C-term.	Exon4(4)	c.1326C > T	p.T442T[d]	7.0%		**R7**, 17, **25**
SNP		Intron3	IVS3 + 23C > G		7.0%		**R15**, **24**, 34

[a]Nucleotides and amino acids are numbered according to GenBank. [b]Frequency of substitution among 43 Korean patients with classical RTT. [c]Three novel mutations were identified in this study. [d]The nucleotide is substituted, but the amino acid is silent. [e]Restriction enzyme sites for RFLP analysis were created (+) or destroyed (-) by novel mutations and sequence variants of the *MECP2* gene in RTT patients. The description of the sequence variant was based on the work of den Dunner and Antonarakis (2000; 2001). The patients indicated by bold type exhibited two types of nucleotide changes.

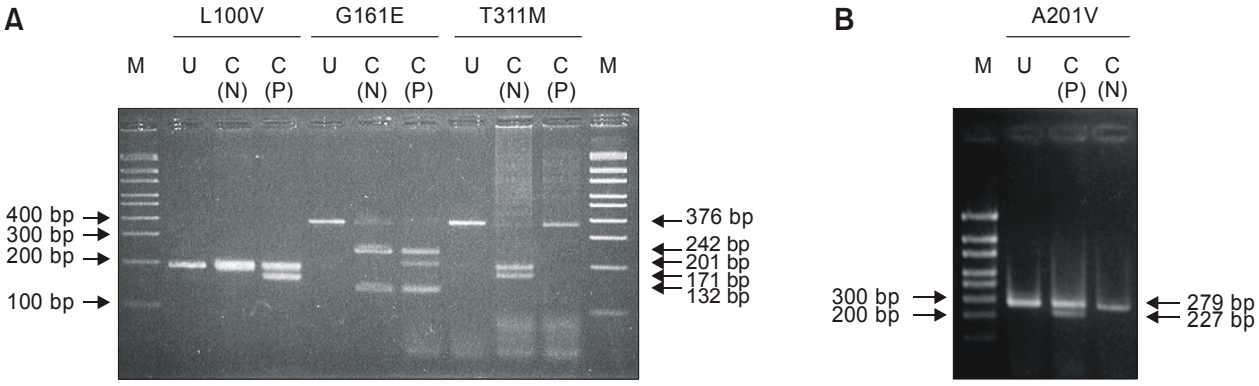

Figure 3. RFLP for novel mutations (G161E, T311M) and unclassified variants (L100V, A201V). Gel electrophoresis of PCR products before (U, uncut) and after (C, cut) the restriction enzyme site. Cut samples are distinguished as normal controls (N) and as mutations in patients (P). (A) PCR product showing the part of exon 3 with a mismatch sequence (lane 1) and exon 4 (lane 4 and 7). The L100V mutation separated the 199 bp PCR product into 170 bp and 29 bp fragments by mutation-induced cleavage at the *Afa* I site. (lane 2 and 3) *Dde* I digestion created a 202 bp and a 39 bp fragment in G161E mutation (lane 5 and 6). T311M mutation identified by loss of the *BsmB* I site (lane 8 and 9). (B) A201V creates a *Bal* I restriction site. Normal controls have 279 bp products and are not cleaved by *Bal* I digestion, while patients with A201V generated 229 bp and 52 bp fragments. Lane M is a 100 bp molecular weight marker. The lower 60 bp products migrated off the gel.

(366 bp) (Figure 3A, lane 9). The novel deletion mutation *p385fsX409* (1153del44bp) was not identified by direct sequence mutational screening of exon 4 in the control samples. All of these base substitutions were absent in more than 100 control individuals. The RFLP and direct sequencing data indicate that these DNA variants are potential disease-causing mutations.

L100V (c.298C > G), one of these previously predicted substitutions, has been reported to be an unclassified variant associated with RTT (Buyse *et al*., 2000). Mismatch PCR was performed to determine whether L100V is also SNP in Korea. After amplification with the mismatch primers, the wild type sequence CCCTACCTGAA was generated. The generated mutant sequence CCG/TACCTGAA created an *Afa* I site. The 199 bp product was cut into pieces of 170 bp and 29 bp (Figure 3A, lane 2 and 3). Additionally, A201V (c.602C > T) has been reported to be a Japanese-specific characteristic because the mutation occurs in the normal Japanese population (Fukuda *et al*., 2005). The 279 bp product with A201V was cut into 227 bp and 52 bp fragments by *Bal* I (Figure 3B, lane 2~10). L100V and A201V were absent in more than 100 normal control subjects and, therefore, were thought to be disease-causing mutations.

Single nucleotide polymorphism (SNP) of the *MECP2* gene

IVS3 + 23C > G (g.C65494G) was observed in only three patients (7%) (Figure 4). The novel SNP accompanied other mutations in two of the patients.

Discussion

In classical cases, the mutation rate approaches 80% with lower rates in atypical cases (30%) (Hoffbuhr *et al*., 2001). There is no clear correlation between the type and position of mutations, although *MECP2* plays a pivotal role in the RTT phenotype (Weaving *et al*., 2003). We identified mutations of the *MECP2* gene in 60.5% (26/43 cases) of the patients (Table 1). The mutational frequency was lower in our results than in previous studies due to the inclusion of patients with various phenotypes of RTT, and not limiting subjects to classical cases, as in previous reports. Most mutations were identified in the functional domains (25/26, 96.2%) MBD, TRD and WDR, and, therefore, probably critically affect the function of *MECP2*. We identified three novel mutations that cause amino acid substitutions (G161E, T311M, *P385fsX409*). G161E was found in MBD, T311M in TRD, and *P385fsX409* in WDR of the C-terminus. The G161E substitution resulted in an alteration of a charge from neutral to negative

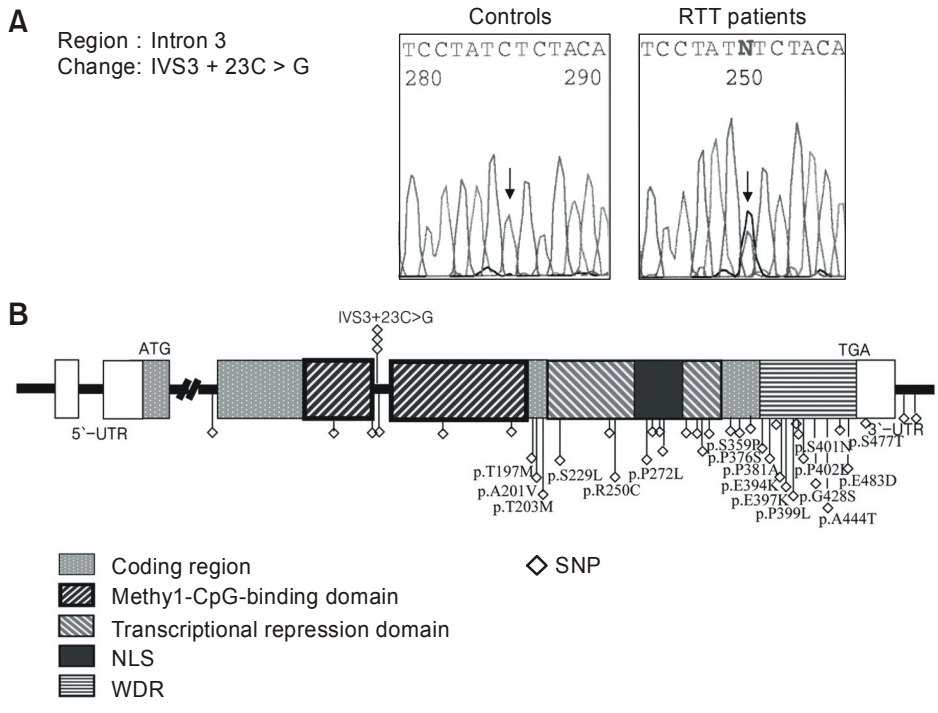

Figure 4. Single nucleotide polymorphism in the *MECP2* gene identified by direct sequence analysis. (A) The substituted nucleotide is indicated by an arrow and the substituted codon is marked by underlining. All sequences are in the sense orientation. (B) Distribution of single nucleotide polymorphism in the *MECP2* gene is identified. Top: The SNP identified in this study, Bottom: SNPs described by others (Buyse *et al*., 2000; Fukuda *et al*., 2005).

and may reduce or abolish methyl-CpG binding. T311M probably causes the hydrophilic amino acid to be replaced by a hydrophobic one. *P385fsX409* results in a reduction or loss of WW domain binding activity. The truncated mutation probably causes loss of the splicing factors FBP11 and HYPC that normally interact with WDR (Buschdorf and Stratling, 2004; Weaving *et al*., 2005). Hence, it may cause defective splicing that is involved with *MECP2*. These mutational results probably alter the properties of the protein. Twenty-two of the mutations (22/26, 84.6%) involved C→T transition at CpG dinucleotides. A clustering of mutations was identified in exon 4, except for L100V (25/26, 96.2%). Our findings strongly indicate that exon 4 should be sequenced first to screen for *MECP2* mutations because the region is a hotspot for *MECP2* mutations in RTT patients. Screening of two (exons 3 and 4) of the four exons will identify almost all gene mutations found in RTT patients, especially in Korean RTT patients.

To predict whether the observed changes were mutations or polymorphism, PCR-RFLP analysis of G161E and T311M was performed. Direct sequencing was used to analyze *P385fsX409*. These mutations were not found in more than 100 control subjects. Therefore, G161E, T311M, and *P385fsX409* are probably disease-causing mutations. Previous studies reported that L100V and A201V are the unclassified sequence and the SNP (Buyse *et al*., 2000; Fukuda *et al*., 2005), respectively. The alleles did not appear in more than 100 Korean control subjects. L100V and A201V were identified as possible disease-causing mutations from RFLP analysis in Korean RTT patients, which is contrary to previous reports. No mutation in *MECP2* was found in the two male RTT patients in our study. Mutations in *MECP2* in most males are lethal (Orrico *et al*., 2000). The infrequent occurrence of RTT in males has been explained by the existence of somatic mosaicism for an RTT-causing *MECP2* mutation (Jellinger, 2003). We identified two silent mutations (F142F, T442T) and an intronic SNP (IVS3 + 23C > G) of the gene. The silent mutations are probably not responsible for the disease phenotype although they were associated with other mutations, except in one patient. IVS3 + 23C > G was observed in only three RTT patients (7%) and was not observed in control subjects. It is possible that IVS3 + 23C > G is a disease-related and Korea-specific SNP for RTT. The novel SNP accompanied other mutations in two of the patients. This polymorphism probably results in phenotypic variability or susceptibility to RTT, even though it is an intronic SNP.

Mutation in *MECP2* is not synonymous with RTT, and RTT is not always caused by an identifiable mutation in *MECP2* (Miltenberger-Miltenyi and Laccone, 2003). Recently, studies have suggested a relationship between RTT and *MECP2* in the regulation of UBE3A (Ubiquitin-Protein ligase E3A), GABRB3 (the beta3 subunit of the GABAA receptor) and CDKL5 (Cyclin-dependent kinase-like5) expression (Fan G and Hutnick L, 2005; Milani *et al*., 2005; Samaco *et al*., 2005; Segawa and Nomura, 2005). In this study, we did not detect any mutations of *MECP2* in seventeen of the patients. These cases should be analyzed for mutations of other candidate genes, followed by functional analysis of these genes, as well as *MECP2*.

PCR-RFLP is an essential step in determining whether the observed changes to the *MECP2* gene are mutations or polymorphisms. Disease-causing mutations and polymorphisms are important for diagnosing RTT in Koreans. The experimental procedures used in this study should be considered for molecular biologic diagnosis in the clinical field.

Acknowledgement

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