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SCIENCE DIRECT
Gene 373 (2006) 83–89
GENE
SECTION
EVOLUTIONARY GENOMICS
www.elsevier.com/locate/gene

The X-linked methyl binding protein gene *Kaiso* is highly expressed in brain but is not mutated in Rett syndrome patients

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Received 10 November 2005; received in revised form 16 January 2006; accepted 17 January 2006
Available online 13 March 2006
Received by M. D'Urso

Abstract

Rett syndrome (RTT; OMIM 312750) is an X-linked dominant neurological disorder, which affects mostly females. It is associated with mutations of the MECP2 gene, codifying for a methyl-CpG DNA binding protein of the MBDs family, sharing the common *M*ethyl *B*inding *D*omain. MeCP2 binds single methylated CpG pair and brings transcriptional silencing to the substrate DNA templates. However, around 5–10% of clinically well defined RTT patients do not show any mutations in this gene. Several hypotheses have been postulated to clarify the remaining unexplained RTT cases. We pointed our attention on *Kaiso* gene. This gene is localized in the Xq23 region and codifies for a protein acting as a methyl-CpG binding protein by using three zinc-finger domains: for this reason it is not strictly related to the MBD family of proteins, even if it may repress transcription of methylated genes as well.

To investigate the potential association of Kaiso disfunction with pathogenesis of Rett syndrome, we approached the analysis at two different levels. Primarily, we performed an itemized murine brain expression analysis of *Kaiso* gene. Expression data and localization made it an excellent candidate as additional causative gene for MECP2 negative, classical RTT patients. On the bases of this data a detailed mutational analysis of 44 patients from Spanish, UK, and Italian archives has been performed to the coding region of *Kaiso*. No mutation was found while a very frequent polymorphism was identified and characterized. Our study suggests that this gene is not implicated in the RTT molecular pathogenesis, but additional analyses are needed to exclude it as causative gene for X-linked mental retardation disorders.

Keywords: X chromosome; Mutational analysis; MECP2; MBD family; XLMR

Abbreviations: RTT, Rett; MECP2, methyl-CpG-binding protein 2; MBD, methyl binding domain; CDKL5, cyclin-dependent kinase-like 5; PCR, polymerase chain reaction; HDAC, histone deacetylase; DPR, deletion prone region; BTB/POZ, broad complex-tramtrack-bric a brac/Poxviruses and zinc fingers; STR, short tandem repeat; SNP, single nucleotide polymorphism; XLMR, X-linked mental retardation; DLX5, distal-less homeo box 5; BDNF, brain-derived neurotrophic factor; MRX, non-syndromic X-linked mental retardation.

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doi:10.1016/j.gene.2006.01.015

1. Introduction

Rett syndrome (RTT, OMIM 312750) is a neurological disorder that affects almost exclusively females, occurring with a frequency of up to 1/10,000 live female births (Kerr, 1991). After an early period of apparently normal or almost normal development (until 6–18 months of age), this disorder produces a profound mental disability, reduction in speech and purposeful hand movements and reduced brain growth. Besides, in Rett patients functional impairment of many cerebral regions, such as the cerebral cortex, basal ganglia, limbic system, cerebellum, brainstem, spinal cord, peripheral and autonomic nervous system, has been found (Engerstrom and Kerr, 2001). The RTT locus has been recently mapped to Xq28, allowing subsequent identification of mutations in the MECP2 gene, which codes for a methyl-CpG-binding protein type 2 (Amir et al., 1999). The MeCP2 protein is capable of binding to methylated DNA preferably in $CGN_{3-8}(A/T)_{\geq 4}$ context (Klose et al., 2005) and has been shown to mediate transcriptional repression by recruiting a corepressor complex containing Sin3A and HDACs (Jones et al., 1998; Nan et al., 1998). The discovery that RTT is caused by mutations in the MECP2 gene provided strong evidence that epigenetic silencing is crucially important for the proper functioning of neuronal cells both in humans and in mice (Chen et al., 2001; Guy et al., 2001).

Approximately 75–80% of RTT females have been found to carry heterozygous mutations in the X-linked MECP2 gene (Cheadle et al., 2000; Vacca et al., 2001a,b; Wan et al., 1999). Large deletions in a deletion prone region (DPR) of the MECP2 gene have been reported in an additional 7% to 16% of the patients analyzed (Laccone et al., 2004) which account for a part of the remaining non-mutated RTT cases. Additionally, MECP2 mutations have been found in male patients with recessive X-linked mental retardation and spasticity (Meloni et al., 2000), and in roughly 2% of patients with nonspecific X-linked mental retardation (Couvert et al., 2001; Orrico et al., 2000).

The data indicate that the small proportion of RTT patients in which no MECP2 mutation has yet been found would carry mutations elsewhere, in related gene. This is indeed the case of the cyclin-dependent kinase-like 5 gene (CDKL5) located in Xp22, whose product interacting with MeCP2 (Mari et al., 2005) is mutated in patients affected by an RTT-like phenotype and early onset seizures variant of RTT (Tao et al., 2004; Weaving et al., 2004, 2005).

Additional candidate genes may presumably share: (i) a similar localization on the X chromosome and (ii) striking brain expression and MeCP2 related cellular functions.

KAISO was first isolated through its ability to interact with the Armadillo-repeat catenin p120 (Daniel and Reynolds, 1999). The interaction was surprising as p120-catenin associates with cadherins at the cell membrane, whereas KAISO behaves as a DNA binding protein. This raised the possibility that the p120-catenin/KAISO pair may functionally resemble the β-catenin/LEF/TCF system by participating in the transmission of extracellular signals from the cell membrane to the nucleus, where KAISO could act as a regulator of target genes (Anastasiadis and Reynolds, 2001). The p120-catenin-binding partner KAISO is a bi-modal DNA-binding protein that recognizes both a sequence-specific consensus and methylated CpG dinucleotides (Daniel et al., 2002). KAISO-mediated repression of the *Xenopus* genes *xWnt11* and *Siamois* appears to be DNA methylation-independent (Park et al., 2005) but KAISO has also been shown to repress transcription of methylated genes (Prokhortchouk et al., 2001; Yoon et al., 2003). *Kaiso* has been mapped on Xq23 chromosomal region and is subject to X inactivation (Carrel and Willard, 2005). Thus, both its role as repressor of methylated substrates and its localization suggest that *Kaiso* fits all formal requirements to be an additional RTT causative gene.

Here we report data on in situ RNA hybridization in adult mouse brain suggesting that *Kaiso* is expressed in neocortex, amygdala, piriform cortex, endopiriform nuclei, hippocampus, brainstem nuclei and cerebellum.

To further investigate a possible role of KAISO in the pathogenesis of RTT, we also developed a PCR based strategy to subdivide the coding region of *Kaiso* into six sub-regions (two of which are overlapping), analyzed by direct sequencing of the amplified products from 44 MECP2 negative RTT patients and 53 healthy controls.

2. Materials and methods

2.1. In situ hybridization analysis

Immediately after sacrifice, a mouse (C57Bl/6, adult male) was decapitated and its brain quickly frozen in liquid nitrogen vapor. Frontal 20 μm cryostat sections were collected on sterile poly-L-lysine coated glass slides and fixed for 5 min in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4 °C. After rinsing in PBS, the sections were acetylated with 0.25% acetic anhydride (pH = 8) for 10 min at room temperature (RT), rinsed again and dehydrated in ascending ethanol series (70%, 95%, 100%) for 2 min in each. The slides were then placed in chloroform for 5 min and finally in 100% and 95% ethanol. Air-dried sections were covered with hybridization buffer containing RNA probe to *Kaiso*. A part of mouse *Kaiso* cDNA (corresponding to 490–672 aa) was cloned into pGEM-T-Easy vector. Transcription originated from T7 promoter produced antisense and Sp6 produced sense RNA probes. The probes were synthesized by in vitro transcription and labeled by incorporation of digoxygenin-UTP according to the manufacturer's protocol (DIG RNA labeling mix, Roche). The probe concentration in hybridization buffer was 0.5 μg/ml. Hybridization buffer contained 50% formamide, 4× SSC, 8% dextran sulfate, 1× Denhardt solution, 0.5 mg/ml denaturated salmon sperm DNA and 0.25 mg/ml tRNA from baker's yeast. The sections were covered with parafilm pieces, placed into a humidified chamber and incubated at 50 °C overnight. On the next day, the slides were washed several times in SSC buffer, treated with RNase A (20 μg/ml) at 37 °C for 30 min, washed in SSC buffers of descending concentrations (2× to 0.1×) at

ascending temperatures (RT to 55 °C) and incubated for 30 min with blocking reagent (Roche) at RT in Tris buffered saline (pH=7.4) containing 0.1% Triton X100 (TBST). After that, the sections were incubated for 4 h at RT with antibodies to digoxygenin (Roche), 1:250 in TBST containing 3% sheep serum. Following incubation, the slides were washed in TBST and placed in STM buffer (Tris buffered saline containing magnesium chloride, pH=9.5). NBT/BCIP stock solution (Roche) was added to the buffer (0.2 ml/10 ml) and the staining was allowed to develop overnight at RT in darkness. The following day, slides were coverslipped with aqueous mounting medium (Merck). The staining was performed on brain slides derived from three independent C57/Bl6 animals. Representative results from one animal set are shown.

The sections were analyzed under microscope (Olympus BX 51) and digitized with Nikon DMX1200 camera. Mouse brain structures were identified using stereotaxic mouse brain atlas (Franklin and Paxinos, 1997).

2.2. Mutational analysis

All the patients showed classical phenotype and after the signature of an informed consent, they have been subjected to mutational analysis of the coding region of *Kaiso* gene. 39 sporadic patients came from Spain; 2 sporadic cases were Italian; 3 from UK archives, 2 of which were half sisters. Among the 53 healthy controls, 33 were females and 20 males.

All PCRs were carried out using AmpliTaq and AmpliTaq Gold DNA polymerase (Perkin Elmer) with buffer recommended by the manufacturer, in the presence of 50 ng of genomic DNA, 0.2 mM dNTP, 0.25 μM (fragments 3, 4, 5 and 6) or 0.5 μM (fragments 1 and 2) oligos, in a final volume of 10 μl. In Table 1, we report the primer sequences we used for the analysis. All the amplifications were performed using a Robocycler® Gradient 96 (Stratagene). Amplified fragments were purified using the QIAquick PCR purification kit (QIAGEN) and sequenced on both strands, by using the same PCR primers with fluorescent-dye terminators on an ABI377 automatic sequencer.

3. Results

3.1. Kaiso mRNA distribution in mouse brain

Given the role in transcriptional repression and its peculiar chromosomal localization, *Kaiso* gene may be an interesting candidate gene for the MECP2 negative RTT patients. As a first step to verify this hypothesis, we performed a thorough expression analysis of the gene in the central nervous system of the adult mouse.

Kaiso expression was detected throughout the whole brain by antisense in situ hybridization. Neurons with cytoplasm labeling were found in neocortex, with more pronounced staining in layer III and deeper layers (Fig. 1A). Also in the lateral amygdaloid nucleus and in basolateral amygdaloid nucleus *Kaiso* signal was detected (Fig. 1B). In the hippocampus, labeled neurons were detected in pyramidal cell layer of CA2 and CA3 fields as well as in single cells of polymorph layer of the dentate gyrus (Fig. 1C). In the cerebellum cortex, nearly all Purkinje cells were intensively stained, whereas only solitary cells were found in the granule layer (Fig. 1D). Within main olfactory bulb, strongly labeled mitral cells were found in the mitral cell layer and weaker labeled periglomerular cells in glomerular layer (Fig. 1E). Besides the forebrain, strongly stained neurons were detected in multiple brainstem nuclei: in the complex of vestibular nuclei; in nuclei of V (trigeminal) and VII (facial) cranial nerves; in several pontine nuclei (Fig. 1F).

3.2. Mutational analysis of Kaiso coding region

Kaiso gene is composed by two exons. The ATG is localized in the 5′ region of exon 2, the stop codon is positioned in the middle of exon 2, while a CpG island spans exon 1 (Fig. 2). The complete mRNA is 5225 bp long, while the protein is composed by 672 amino acids (data not shown). The N-terminus of

Table 1
Primer sequences and PCR amplification conditions of *Kaiso* gene

Primer name	Nucleotidic sequence (5′→3′)	Fragment name and size		PCR settings		
				Denaturation	Annealing	Extension
X KAISO217	TCTCTAATCCTCTCCCATCC	Fragment 1, 517 bp	Time (s), temperature (°C), 35 cycles	30	45	45
X KAISO714	ATCTTGGGCTTCATCTTTTG			95	60	72
X KAISO668	CCTTTACCTCCTGATTCTGG	Fragment 2*, 545 bp	Time (s), temperature (°C), 35 cycles	30	60	60
X KAISO1193	CTGTTTGGTGTCTGCTGATT			95	56	72
X KAISO1160	CATATGCCCTCTTCAATCAA	Fragment 3, 527 bp	Time (s), temperature (°C), 35 cycles	30	45	45
X KAISO1667	ATACGTTTGTTTGCCATCTC			95	56	72
X KAISO1621	AGGGGAGGCCAGACTTGAGA	Fragment 4*, 290 bp	Time (s), temperature (°C), 35 cycles	30	30	30
X KAISO1891	GATACCTTCGCTCCCCTGTG			95	56	72
X KAISO1823	TATCCGTGCCGTTACTGTGA	Fragment 5*, 515 bp	Time (s), temperature (°C), 35 cycles	30	30	30
X KAISO2318	ATCTTCTGAAACCCCTGCCC			95	58	72
X KAISO570U	CAGGGCAGTTATTAGGAGTGAA	Fragment 6, 513 bp	Time (s), temperature (°C), 35 cycles	30	45	45
X KAISO1063L	ATGTGTTGGAGCTGAAGATACC			95	62	72

Asterisk (∗) denotes the use of AmpliTaq Gold (Perkin Elmer).

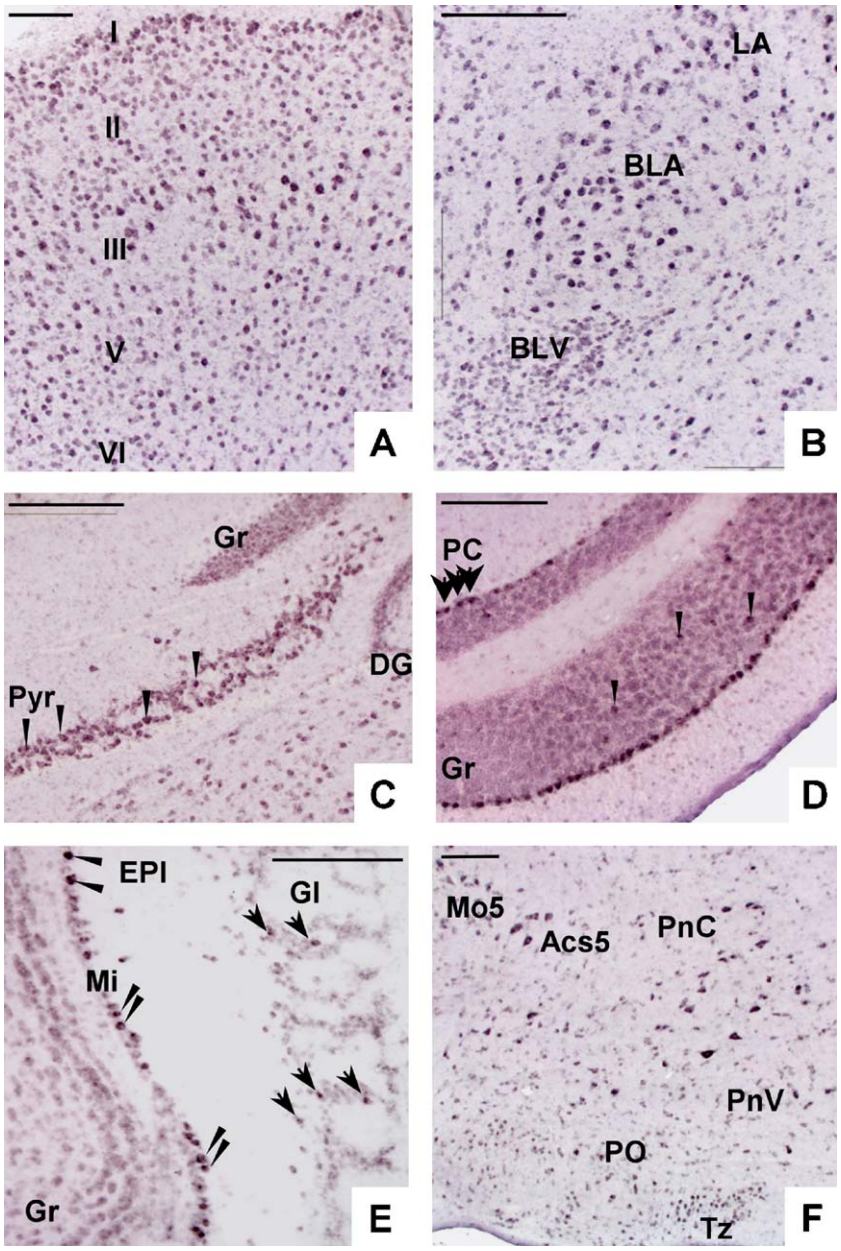

Fig. 1. *Kaiso* expression in the central nervous system. (A) *Kaiso* expression in the neocortex. I–VI: neocortex layers. (B) *Kaiso* expression in the amygdala. LA: lateral amygdaloid nucleus; BLA: basolateral amygdaloid nucleus, anterior part; BLV: basolateral amygdaloid nucleus, ventral part. (C) Antisense probe labeling of *Kaiso* in the hippocampus. Arrowheads show labeled cells in the pyramidal cell layer of CA3 field. DG: dentate gyrus; Gr: granular layer of the dentate gyrus; Pyr: pyramidal cell layer. (D) *Kaiso* expression in the cerebellum. Arrows show Purkinje cells (PC); arrowheads: labeled cells in the granule layer (Gr). (E) Antisense probe labeling of *Kaiso* in the main olfactory bulb. Arrowheads: labeled mitral cells in the mitral cell layer; arrows: labeled periglomerular cells in glomerular layer. Epl: external plexiform layer; Gl: glomerular layer; Gr: granule layer; Mi: mitral cell layer. (F) *Kaiso* expression in brainstem nuclei. Acs5: accessory trigeminal nucleus; Mo5: motor trigeminal nucleus; PnC: pontine reticular nucleus caudal; PnV: pontine reticular nucleus ventral; PO: paraolivary nuclei; Tz: nucleus trapezoid body. Bars = 0.2 mm.

KAISO protein contains BTB/POZ domain, while at C-terminus of protein three zinc finger motifs are present.

In order to understand if KAISO may have a direct role in the pathogenesis of RTT, we performed a detailed mutational analysis by direct sequencing of 44 RTT MECP2 negative patients and 53 controls.

The primers we designed covered the entire coding region: our PCR based strategy subdivided the coding region of *Kaiso* into six sub-regions (two of which are overlapping) (Fig. 2).

This detailed analysis did not show any mutation nor polymorphism in fragment 1 (517 bp), codifying the conserved BTB/POZ domain of the protein. Analysis of fragment 3 (527 bp) did not reveal any nucleotide change, not even in fragment 4 (290 bp) nor in fragment 5 (515 bp).

The analysis of the 545 bp fragment 2 pointed to the existence of a STR (short tandem repeat) polymorphism where a GAT repeat (Fig. 2A), producing a “poly Asp” stretch, could vary from a number of six to seven; it is located at position 189 of the amino acidic sequence (EMBL Acc. AAH42753). We

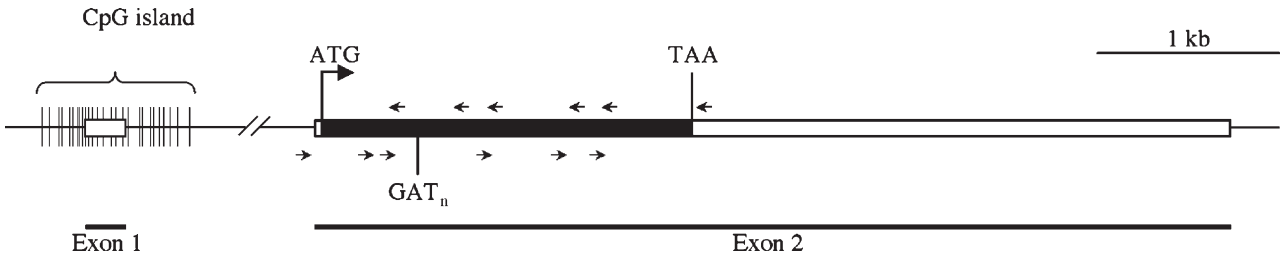

Fig. 2. Schematic diagram illustrating genomic organization of human *Kaiso* gene. White boxes indicate, respectively, 5′ and 3′ untranslated regions. Black box represents coding region, localized in the exon 2. Positions of two exons are indicated by continuous lines. Vertical lines around the exon 1 represent the CpG island. Start and stop codons are showed, as well the position of GAT_n polymorphism. Black arrows represent primers used to carry out mutational analysis.

characterized the genotypic and allelic frequencies for this polymorphism; the results are summarized in Table 2. A polymorphism (C/T, rs2285406) reported in the SNP database (http://www.ncbi.nlm.nih.gov/SNP/) was not detected in our population, thus suggesting that it has a very low frequency.

4. Discussion

Mental retardation is the main neurological disorder in children, reaching the 2–3% of live births in developing countries (Ropers and Hamel, 2005). Since mental retardation is far more common in males than in females, the role of X-linked loci in the pathogenesis of the disorder has been firmly considered. Extensive linkage pointed out few regions for syndromic XLMR, especially on the short arm of the X chromosome, on the long arm pericentromeric region and on the telomeric Xq28.

Among syndromic XLMR, Rett syndrome, a severe disease leading to the cessation and regression of psychomotor development in 1/10,000–15,000 females, shows mutations in the gene encoding the transcriptional repressor MeCP2 (Amir et al., 1999). However, MECP2 is mutated in a wide spectrum of disorders, such as encephalopathy, progressive spasticity, and non-syndromic XLMR (Couvert et al., 2001). MECP2 mutations, in the two described splicing isoforms of this gene, satisfy the larger part of the described RTT cases, even if the familial cases and those having atypical features have still an unknown pathogenesis. Recently, mutations on the X-linked, cyclin dependent protein kinase like 5 (CDKL5) gene have been identified in atypical cases of RTT with early onset of seizures (Mari et al., 2005; Tao et al., 2004; Weaving et al., 2004, 2005).

The possibility that other X-linked causative genes for RTT may exist prompted us to search for additional causative loci, especially for those classical and familial cases with no mutations in MECP2 gene.

Kaiso gene codifies for a transcriptional factor, localized on the X chromosome at Xq23 region. It acts mainly as repressor and is capable to bind methylated DNA (Prokhortchouk et al., 2001).

Our analysis of mRNA distribution in adult mice brains revealed uniform *Kaiso* labeling throughout the whole brain, suggesting that the gene was expressed constitutively. Though no special investigation was conducted to detect phenotype of the expressing cells, for some brain regions we were able to conclude that the expression was neuronal rather than glial. Intensive staining was found in the Purkinje cells in the cerebellar cortex, in the mitral and periglomerular cells of the olfactory bulb, in the pyramidal cells of the hippocampus, in lateral and basolateral amygdaloid nuclei, thus proving that the *Kaiso* mRNA was located in the neuronal cytoplasm.

Kaiso was thus considered a good candidate either for chromosomal location and function; our mutational studies however suggest that it might not be implicated in the molecular pathogenesis of the Rett syndrome, even if non-coding regions of *Kaiso* were not investigated in our analysis. Noteworthy, an outcome of our gene sequencing analysis was the identification of a variable number (6 to 7) of Asp codon trinucleotide repeat in the coding region of *Kaiso*. Aspartic acid belongs to the residues often found in single amino acid repeats (SAARs, Depledge and Dalby, 2005). Moreover, runs of identical amino acids have been frequently linked to transcriptional regulators and proteins involved in (neuro)-development (Karlin et al., 2002). When the number of amino acids in the repeat exceeds the gene-specific threshold number, a disease may develop (i.e. polyGln neurodegenerative disorders). Even if we did not find an Asp triplet expansion in RTT patients with respect to healthy controls, we could suggest to search for such an amplification in other X-linked syndromic or non-syndromic mental retardation. The question of what is the cause for classical and/or familial Rett syndrome in the absence of MECP2 mutations is therefore still open. To completely exclude *Kaiso* gene as causative for RTT, additional analyses should be addressed. A possibility is that either another gene or disregulation of MECP2 mRNA by mutations in promoters/enhancers, splicing sites, untranslated 3′ and 5′ ends of MECP2 (the very large 3′ end makes the analysis too difficult) may explain the remaining RTT cases. Indeed, even if MeCP2 plays a very important role in the pathogenesis of RTT, it is not the only gene involved. Very few target genes,

Table 2
Genotypic and allelic frequencies of STR polymorphism in 44 RTT patients and 53 healthy controls

Genotypes	Patients	Controls
$X^{(GAT)6}/X^{(GAT)6}$	4/44 (9.1%)	4/33 (12.1%)
$X^{(GAT)7}/X^{(GAT)7}$	24/44 (54.5 %)	15/33 (45.4%)
$X^{(GAT)6}/X^{(GAT)7}$	16/44 (36.4%)	14/33 (42.4%)
$X^{(GAT)6}/Y$	–	7/20 (35%)
$X^{(GAT)7}/Y$	–	13/20 (65%)
Allelic frequency of $X^{(GAT)6}$	24/88 (27.3%)	29/86 (33.7%)
Allelic frequency of $X^{(GAT)7}$	64/88 (72.7%)	57/86 (66.3%)

including the transcriptional repressor of neuron specific genes, Hairy2a (Stancheva et al., 2003) and the brain derived growth factor BDNF (Chen et al., 2003; Klose et al., 2005; Martinowich et al., 2003) have been identified. Recently, the relaxation of imprinting for DLX5 gene, both in MeCP2 null brains and in lymphoblastoid cells from Rett patients, has been demonstrated. In this case it seems that the lack of MeCP2 alters the silent chromatin looping at DLX5 locus (Horike et al., 2005). The identification and the analysis of all the MeCP2 downstream genes and/or genes involved in brain maintenance will be of significance in order to obtain an overall clearer picture of the onset of this syndrome.

Recent results suggested that targeted disruption of *Kaiso* gene in mice did not cause any neurological disorders (Prokhortchouk et al., 2006). This fact supports the main message of our work that *Kaiso* gene is not involved in pathogenesis of Rett syndrome.

Acknowledgements

The authors acknowledge Ms. A. Aliperti for expert secretarial assistance. The financial contribution of Telethon Grant GGP02308 and the European Commission Network of Excellence "SAFE" Contract LSHB-CT-2004-503243 to MDE are gratefully acknowledged. MH is a Medical Advisor to the Rett Syndrome Association UK, and is grateful for the support provided to her by the Association. EP gratefully acknowledges the Russian Fund for Basic Research Grant 02-04-48457. EP was supported by the Wellcome Trust Award GR067436MA, by the Rett Syndrome Research Foundation (http://www.rsrf.org) and by FASI funding (Contract No. 02.442.11.7235). MP also acknowledges the Marato of TV3 for the help received.

FDR is recipient of a fellowship from European Commission Network of Excellence "SAFE". MV is recipient of a Post-Doctoral fellowship from University of Padua.

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