



Variant Rett syndrome in a girl with a pericentric X-chromosome inversion leading to epigenetic changes and overexpression of the *MECP2* gene



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ABSTRACT

Rett syndrome is a neurodevelopmental disorder caused by mutations in the *MECP2* gene. We investigated the genetic basis of disease in a female patient with a Rett-like clinical. Karyotype analysis revealed a pericentric inversion in the X chromosome –46,X,inv(X)(p22.1q28), with breakpoints in the cytobands where the *MECP2* and *CDKL5* genes are located. FISH analysis revealed that the *MECP2* gene is not dislocated by the inversion. However, and in spite of a balanced pattern of X inactivation, this patient displayed hypomethylation and an overexpression of the *MECP2* gene at the mRNA level in the lymphocytes (mean fold change: 2.55 ± 0.38) in comparison to a group of control individuals; the expression of the *CDKL5* gene was similar to that of controls (mean fold change: 0.98 ± 0.10). No gains or losses were detected in the breakpoint regions encompassing known or suspected transcription regulatory elements. We propose that the de-regulation of *MECP2* expression in this patient may be due to alterations in long-range genomic interactions caused by the inversion and hypothesize that this type of epigenetic de-regulation of the *MECP2* may be present in other RTT-like patients.

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1. Introduction

Rett syndrome (RTT) is a neurodevelopmental disorder characterized by an early regression followed by stagnation in growth and development, leading to mental retardation, stereotypies and motor apraxia that may be associated with epilepsy and with dysautonomic features, including disturbed breathing, sleep and gastrointestinal motility (Hagberg et al., 1983; Rett, 1966). RTT is caused by mutations in the *MECP2* gene, encoding a methyl-CpG

binding protein thought to be involved in transcriptional regulation (Amir et al., 1999; Hoffbuhr et al., 2001; Philippe et al., 2006). Mutations in this gene are present in more than 90% of patients that fulfill the diagnostic criteria, and with lesser frequency in patients with so-called variant forms of the disease, that do not comply with all these criteria (Amir et al., 1999; Hoffbuhr et al., 2001; Philippe et al., 2006).

The most frequent mutations in *MECP2* are point mutations, small insertions or deletions. The genotype of patients without one of these mutations has also been investigated and some cases with gross rearrangements involving the *MECP2* gene were found, usually large deletions. In boys, *MECP2* duplication is the rare cause of a severe encephalopathy with recurrent infections, severe mental retardation, microcephaly and epilepsy (Ramocki et al., 2009; Van Esch et al., 2005). *MECP2* duplication has also been described in a

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girl with a preserved-speech variant of RTT and in another with nonspecific mental retardation (Ariani et al., 2004; Makrythanasis et al., 2010). Recently Bijlsma et al. reviewed *MECP2* duplication in eleven females; five were from their study and six had been previously reported. Intellectual disability was a prominent feature while hand stereotypies, epilepsy, dysmorphism and recurrent infections were less frequent (Bijlsma et al., 2012). Overexpression of normal *MeCP2* has also been shown to cause a progressive neurological disorder in a mouse model, showing that the nervous system requires a very precise dosage of this protein for proper functioning (Collins et al., 2004).

Here, we describe a patient with a RTT-like clinical presentation in whom we found no mutations in the *MECP2* gene by direct sequencing, nor changes in exon dosage. Karyotype analysis, however, revealed a pericentric inversion in the X chromosome 46,X,inv(X)(p22.1q28). This inversion is accompanied by epigenetic changes in the form of decreased DNA methylation levels within the 5' regulatory region of *MECP2* gene, and led to a significantly increased expression of the gene, in spite of its normal dosage, through a mechanism that may involve relatively distant regions of the X chromosome.

2. Materials and methods

2.1. Patient assessment

The proband was ascertained and assessed through the Hospital Dona Estefânia (Centro Hospitalar de Lisboa Central) and *MECP2* study was requested at the Molecular Diagnostic Service at Life and Health Sciences Research Institute (ICVS) – School of Health Sciences, University of Minho, Braga, Portugal. Informed consent was obtained from the child's parents for blood sampling and genetic analyses. Genomic DNA was extracted from peripheral blood using the Puregene DNA isolation kit (Gentra, Minneapolis, MN).

2.2. *MECP2* mutation detection

The coding region and exon-intron boundaries of the *MECP2* gene were amplified by PCR and sequenced on an automated DNA-sequencer (ABI-3130 Genetic Analyzer, Applied Biosystems, Life technologies).

Copy-number analysis of the *MECP2* gene was performed by multiplex ligation-dependent probe amplification (MLPA) (MRC-Holland, Amsterdam, Netherlands) using SALSA P015 kit and quantitative PCR analysis (qPCR). For MLPA, PCR reactions were carried out using C1000 – Thermal Cycler (BioRad) thermocycler according to the manufacturer's instructions. Products were separated in a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using ROX-500 as internal pattern. The data was analyzed for copy-number differences with Coffalyser software (MRC-Holland, Amsterdam, Netherlands). Primers for qPCR were designed using Primer3Plus software (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) and taking into account standard recommendations for qPCR primer development (Jovanovic et al., 2003). Four sets of primers were designed for the *MECP2* gene (ENSG00000169057) within the coding region of exon one, two and three and in the 3'UTR non-coding region of exon four. The reference genes used were *SDC4* (ENSG00000124145) and *ZNF80* (ENSG00000174255) localized in the 20q12-q13 and 3p12 regions, respectively. qPCR reactions were carried out in a 7500-FAST Real Time PCR machine (Applied Biosystems, Foster City, CA, USA) using Power SYBR Green® (Applied Biosystems). The specificity of each of the reactions was verified by the generation of a melting curve for each of the amplified fragments. The primer efficiency was calculated by the generation of a standard curve fitting the accepted normal efficiency percentage. Quantification was

performed as described elsewhere (Hoebeek et al., 2005). Ct values obtained for each test were analyzed in DataAssist™ software (Applied Biosystems, Foster City, CA, USA).

2.3. X chromosome inactivation

The X-inactivation assay (XCI) was carried out by analysis of the HUMARA (human androgen receptor) locus, which is a highly polymorphic CAG repeat with a very near HhaI enzyme restriction site, according to the described protocol (Jovanovic et al., 2003). The products were separated on an automated DNA-sequencer (ABI-3130 Genetic Analyzer, Applied Biosystems, Life technologies) the allele peak height serving as the semi-quantitative measure of the amount of PCR product amplified from each allele. The samples were defined as having a deviated XCI pattern if they presented a corrected allele ratio out of the 0.33–3 range.

2.4. Conventional karyotyping and FISH

Standard karyotype analysis was performed using cells cultured from peripheral blood leukocytes, and FISH analysis was undertaken using two distinct probes covering the Xq28 region: RP4-671D9 (153286964–153386210) and RP11-617G06 (153110141–153283527) (BlueGnome, UK).

2.5. Molecular karyotyping

The aCGH analysis was performed on a human genome CGH Agilent 180K custom array designed by the Low Lands Consortium (LLC, Dr. Klas Kok) in order to be used in the analysis of children with ID/DD (AMADID:023363; Agilent, Santa Clara, CA). DNA labeling was performed using the ENZO Labeling Kit for Oligo Arrays (Enzo Life Sciences, Inc.). As reference DNA we used Kreatech's MegaPoll Reference DNA (Kreatech Diagnostics). Arrays were then hybridized using the Agilent SurePrint G3 Human CGH Microarray Kit. Data was extracted with the Agilent Feature Extraction (FE) Software v10.5 using default settings for CGH hybridizations. Image analysis was performed using the across-array methodology described previously (Buffart et al., 2008). CGH data was analyzed using Nexus Copy Number 6.0 software with FASST2 Segmentation algorithm. The custom 1 M Agilent array was designed using the eArray web tool (<https://earray.chem.agilent.com/earray/>). The maximal amount of probes available was selected for the breakpoint regions (Xq22.1 and Xq28). DNA labeling, hybridization and data extraction were performed in the same way as for aCGH 180K (described above). CGH data was analyzed using Nexus Copy Number 6.0 software with the FASST2 Segmentation algorithm.

2.6. mRNA expression analysis

Total RNA was isolated from leucocytes using TRIZOL (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. First-strand cDNA, synthesized using iScript™ cDNA Synthesis Kit (Bio-rad), was amplified by quantitative reverse-transcriptase PCR (qRT-PCR) using QuantiTect SYBR Green PCR Kit (Qiagen). Human *MECP2*, *CDKL5* and *HPRT* primers were used for relative quantification analysis. *MECP2* (Forward: 5'-GGCGCTCCATCATCCGTGAC-3'; Reverse: 5'-CTGGGGATTGATCAAATACACAT-3'), *CDKL5* (Forward: 5'-CATTCACTGTCTGCACCTCAC-3'; Reverse: 5'-GTTCTCCAGGTCGGGGTGAC-3') and *HPRT* (Forward: 5'-CTAATTATGGACAGGGACTGAACG-3'; Reverse: 5'-CAGTCATAGGAATGGATCTATCA-3'). The expression levels of the genes were normalized to the *HPRT* gene and relative quantification was used to determine the fold change difference between *MECP2*, *CDKL5* and *HPRT*, using the DDCT method, as described before (Pfaffl, 2001). The comparison between the case study

($n = 1$) and controls ($n = 6$) is presented, with the respective t -test analysis for replicate 1. This difference was reproduced for the three replicates.

2.7. Methylation analysis

For epigenetic analysis the EpiTect Methyl II PCR Primer Assay for Human *MECP2* was used where a region of the CpG island 106 (ChrX: 153362568–153363473) was assessed (CpG Island 115358) EPHS115358-1A (Quiagen®) in the index case, mother and 4 healthy female controls. Briefly, 250 ng of DNA from each sample was digested for 6 h at 37 °C degrees. The samples were then amplified by real time PCR using the kit specific *MECP2* primers (EPHS115358-1A) with RT² qPCR SYBR Green ROX master mix in a 7500-FAST Real Time PCR machine (Applied Biosystems, Foster City, CA, USA). Ct values obtained for each sample were analyzed accordingly with the manufacturer's instructions in order to obtain the gene methylation status, as the percentage of unmethylated (UM) and methylated (M) DNA in the input genomic sequence. A t -test analysis was performed for the unmethylated DNA in replicate 1 of the assay where the case study ($n = 1$) is compared with the controls ($n = 4$). This difference was reproduced for the three replicates.

3. Results

3.1. Clinical description

The patient is the second child of healthy, non-consanguineous parents and has no family history of Rett syndrome, intellectual disability or epilepsy. Gestation was uneventful. Delivery, at 39 weeks, was complicated by a difficult forceps extraction. Apgar scores were 8 at 1 min and 10 at 5 min. Birth weight was 3755 g (75th centile), length 50 cm (50th centile) and head circumference 35.5 cm (75th centile). A brain computed tomography (CT) showed a parietal skull fracture and a small underlying epidural hematoma without parenchymatous brain lesions. In the first days, she remained clinically well, and a control CT scan showed total resolution of the hematoma. The child was first seen by us when she was forty days old, after a brief left-sided clonic partial motor seizure. The neurologic examination was normal. Electroencephalogram (EEG) showed focal paroxysmal activity. CT scan and magnetic resonance imaging (MRI) were normal. Phenobarbital was introduced and up to five months she experienced a few more seizures with a similar pattern. Subsequently she remained asymptomatic. The EEG, repeated at five months, was normal. At this time, however, psychomotor development was clearly in the delayed range. The patient had axial hypotonia and her hands infrequently reached the midline. At nine months she did not sit without support, was unable to reach out and pick objects and her lower limbs were slightly spastic. From twelve months on, RTT-like hand stereotypies appeared and became progressively more frequent. She did not acquire at this time any expressive or comprehensive language and did not point to objects. In the period between four and eighteen months head circumference went from the 75th centile to slightly below the 5th centile. Formal developmental evaluation was performed using the Ruth Griffiths scale at the age of four (45 months), revealing a mental age of 15 months and a Global Developmental Quotient of 34%.

At the age of six to seven she was walking with minimal support, hand movements were more coordinated and intentional, she held a pen and scratched, was able to point to some figures in a book and to say six to eight simple words. Currently, at the age of ten, her vocabulary is still limited to eight words, fine and gross motor skills remain at the same level and RTT-like hand stereotypies are still present, although less frequently than before.

Teeth grinding is frequent. Hyperpnea, apnea, insomnia, unprovoked laughing/screaming and autistic features were not reported by the parents. There is no history of recurrent and/or severe infectious diseases. Recently, epilepsy relapsed with brief hypomotor seizures that were controlled with valproate and oxcarbazepine. EEG reveals posterior temporal spikes.

The child is generally happy, smiles quite often and has good visual interaction. She has a divergent strabismus, misalignment and deformity of the teeth, a slight dorsolumbar scoliosis and no dysmorphic features. Her feet are often cold and pale. Neurologic examination is remarkable for the microcephaly, expressionless face suggesting an extrapyramidal hypokinetic disorder, Rett-like hand movements, spastic hypertonia of lower limbs, dyspraxic gait and brisk reflexes with flexor plantar responses.

3.2. *MECP2* mutation analysis and karyotyping

We found no variants in the coding region and intron-exon boundaries of this gene by direct Sanger sequencing, nor changes in exon dosage by MLPA and quantitative PCR analysis (qPCR) for the 4 exons. Karyotype analysis in cells cultured from peripheral blood leukocytes, however, revealed a pericentric inversion in the X chromosome 46,X,inv(X)(p22.1q28) in the patient, which was not present in the mother or father (Fig. 1A). Of notice, the *MECP2* gene is located in cytoband Xq28, and the *CDKL5* gene in Xp22.1. We then performed FISH to test the hypothesis that the pericentric inversion could have displaced the *MECP2* coding sequence or its nearby regulatory regions. We used two probes, one (RP4-671D9) covering the *MECP2* gene and 22998 bp of its 5' region, and the second (RP11-G17G06) covering 173386 bp of its 3' downstream region of the *MECP2* gene (see Fig. 1, panel B and C). The analysis, however, revealed that the *MECP2* gene 5', exonic, and 3' regions had not been separated by the inversion. These results place the breakpoint at Xq28 more proximally than the location of the RP11-617G06 probe (153110141–153283527, hg19 coordinates), meaning that the breakpoint is not occurring within a distance of 176.883 Kb downstream the 3' UTR of the *MECP2* gene. In order to clarify if the inversion lead to any loss or gain in the breakpoints we undertook aCGH analysis on a human genome CGH Agilent 180K custom array (Buffart et al., 2008) and on a high-resolution custom 1 M Agilent array designed so that the maximal amount of Agilent probes available was selected for the breakpoint regions (Xp22.1 and Xq28 where the *CDKL5* and *MECP2* genes are located, respectively) and at the cytobands 14q12 (where the *FOXG1* gene is located). No significant CNVs were found in these regions in both analyses.

3.3. *MECP2* mRNA expression and methylation status

We then investigated by qRT-PCR the expression levels of the *MECP2* (located at Xq28) and *CDKL5* (located at Xp22) genes. Intriguingly, the patient displayed an overexpression of the *MECP2* gene at the mRNA level in the lymphocytes (mean fold change for the three replicates: 2.55 ± 0.38), in comparison to a group of 6 control female individuals; in contrast, the expression of the *CDKL5* gene was similar to that of controls (mean fold change for the three replicates: 0.98 ± 0.10) (Fig. 2A). The X-inactivation assay (XCI), carried out by analysis of the HUMARA locus (Jovanovic et al., 2003), showed that the patient had a balanced pattern of X inactivation, with a ratio of 1/1. To further explore the mechanism leading to the overexpression of *MECP2*, we assessed the methylation status of a portion of the CpG island located in the 5'-regulatory region of the *MECP2* using the EpiTect® Methyl II PCR Assay (Quiagen®). These analyses showed decreased levels of

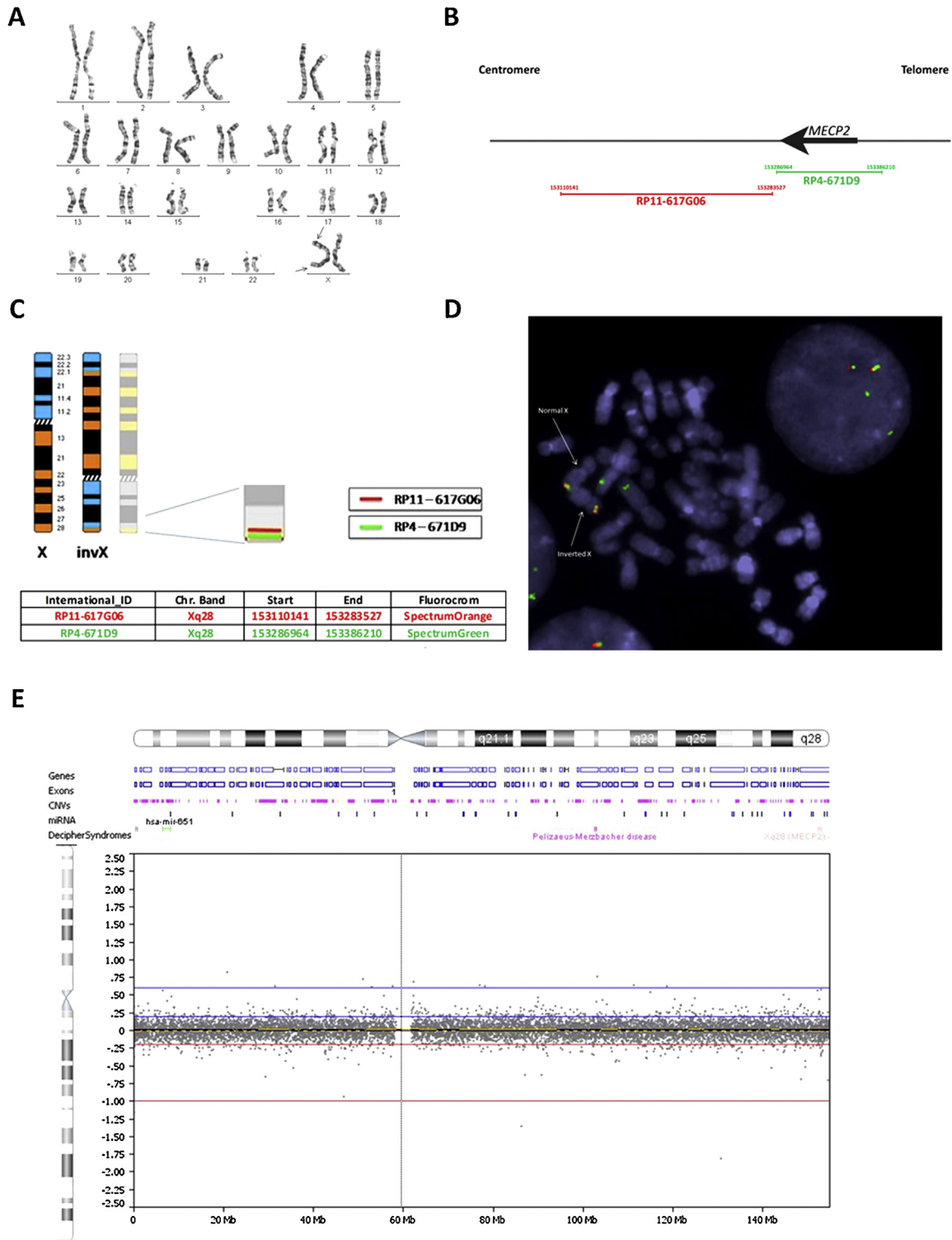


Fig. 1. Cytogenetic and molecular analysis. (A) Karyotype of the patient; (B) schematic representation of the position of the FISH probes relative to the *MECP2* gene; (C) Scheme of the results of the FISH analysis of the patient, undertaken using two distinct probes targeting the Xq28 region: RP4-671D9 (153286964–153386210) and RP11-617G06 (153110141–153283527) (coordinates in hg19); (D) FISH image (arrows indicate the inverted and normal X chromosomes); (E) 180K custom array overview of the X chromosome showing absence of CNVs.

methylation at the assessed CpG site (Fig. 2C), a pattern that is likely associated with the increased *MECP2* gene expression observed in lymphocytes, and which we speculate may also be happening in the nervous system. These findings lead us to speculate that *MECP2* overexpression is the most likely disease causing mechanism in this patient.

4. Discussion

Rett syndrome (RTT) results in a large fraction of patients (more than 90%) from mutations in the *MECP2* gene in patients that fulfill the diagnostic criteria and, in lesser frequency, in patients with so-called variant forms of the disease, that do not comply with all

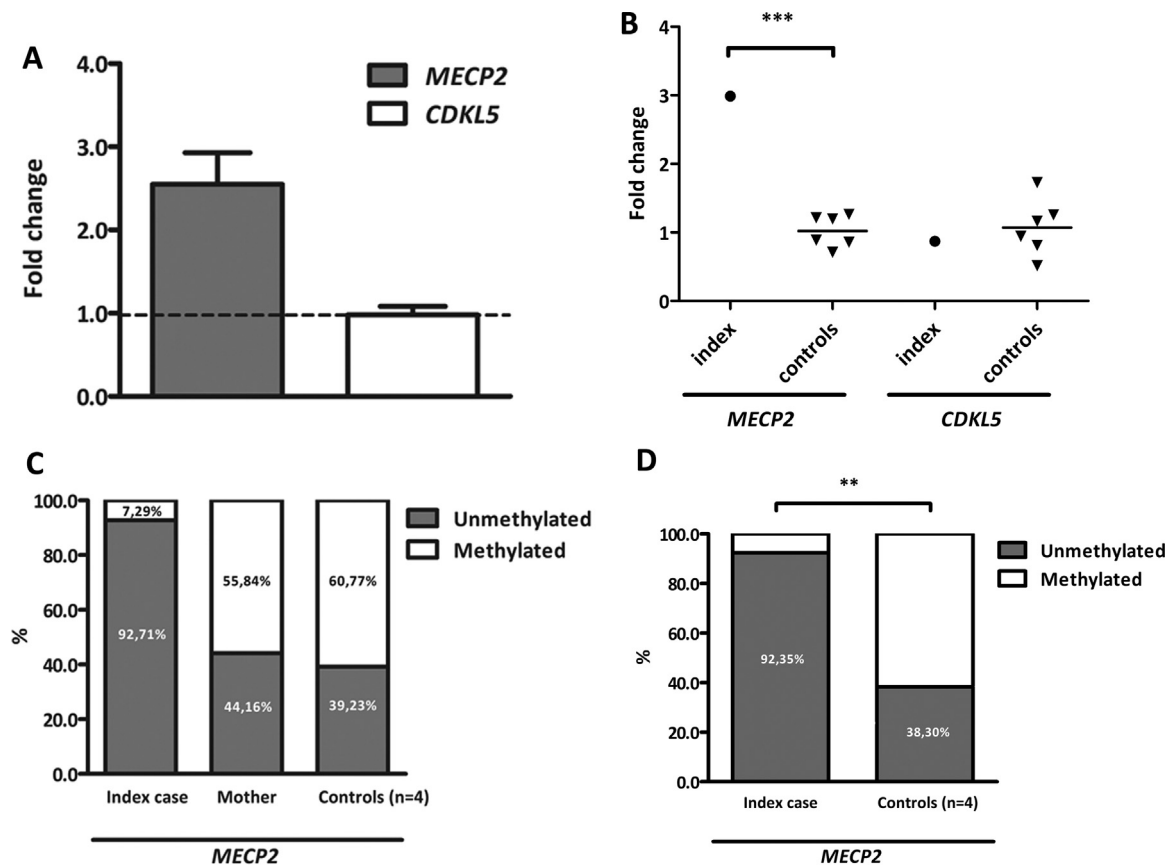


Fig. 2. *MECP2* and *CDKL5* mRNA expression levels and methylation status of *MECP2*. (A) qRT-PCR analysis revealed an upregulation of *MECP2* mRNA levels (mean fold change for the three replicates: 2.55 ± 0.38) in the lymphocytes of the index case in comparison to a group of control female individuals ($n = 4$) (the *HPRT* gene was used as a housekeeping gene); (B) Statistical analysis for *MECP2* and *CDKL5* mRNA expression between the case study ($n = 1$) and controls ($n = 6$) is presented, with the respective t-test analysis for replicate one ($p = 0.000532$; $*** p < 0.001$). This difference was significant for the three replicates. (C) Analysis of the DNA methylation status of the part of the CpG island 106 (ChrX: 153362568–153363473, hg19 coordinates) in the index patient, mother and controls ($n = 4$) using the EpiTect Methyl II PCR Primer Assay for Human *MECP2* (mean values for the three replicates). (D) Statistical analysis (t-test) for percentage of unmethylated DNA for replicate one where the case study ($n = 1$) is compared with the controls ($n = 4$) ($p = 0.002152$; $**p < 0.05$). This difference was significant for the three replicates.

these criteria (Amir et al., 1999; Hoffbuhr et al., 2001; Philippe et al., 2006).

Although our patient does not meet the diagnostic criteria for classic RTT (because she fulfills only two of the four major criteria), her development was normal in the first months of life and later a severe retardation emerged, she has acquired microcephaly, neurologic signs in lower limbs, cold and pale feet, scoliosis, bruxism and, importantly, a persistent and prolonged history of RTT-like hand stereotypies, which are among the most striking clinical features of RTT. With this combination of signs and symptoms she also does not strictly meet diagnostic criteria for variant RTT as published in 2001 (Hagberg et al., 2002) or, more recently, in 2010 (Neul et al., 2010); she does, however, meet the criteria for variant RTT according to the early Hagberg classification (Hagberg and Skjeldal, 1994). The main difference between later and former diagnostic criteria is that the 2001 and 2010 criteria emphasize regression (which is questionable in this case) as a key diagnostic feature of RTT. Therefore, and given this phenotypic resemblance, we were encouraged to study the *MECP2* gene.

Although the *MECP2* gene itself was not mutated nor involved in the chromosomal rearrangement, its expression was altered, and this deregulation might underlie the neurodevelopmental phenotype observed. This phenotype differs from that of male patients with *MECP2* duplications (Van Esch, 2012). Heterozygous females with *MECP2* duplication usually show extreme to complete skewing of X-chromosome inactivation and are asymptomatic or have mainly neuropsychiatric disorders, but severe mental retardation

has been reported (Bijlsma et al., 2012). We were not able to determine the mechanism through which the pericentric inversion is affecting the methylation and the transcriptional regulation of the *MECP2* gene. aCGH analysis, provided no evidence for deletions or duplications encompassing other genes described as mutated in RTT-like phenotypes or a known or suspected regulatory element that could be related with *MECP2* altered expression. Taking these findings in consideration, we hypothesize that the structural alteration of the X chromosome, even without significantly large CNVs, could be disrupting chromosomal domain organization and long-range genomic interactions (Maksimenco and Georgiev, 2014; Robyr et al., 2011), thus influencing *MECP2* epigenetic modifications, including DNA methylation, and consequently modifying its expression. This type of perturbation has been described in fibroblasts of patients with Down syndrome (Letourneau et al., 2014).

In summary, we report the case of a girl with a Rett syndrome-like clinical presentation with a pericentric inversion in the X chromosome 46,X,inv(X)(p22.1q28) that doesn't disrupt the *MECP2* gene but is accompanied by epigenetic changes in the form of decreased DNA methylation levels within the 5' regulatory region of *MECP2* gene, leading to a significantly increased expression of the gene. We speculate that this de-regulation of *MECP2* expression may be a novel mechanism underlying disease in other RTT-like patients who test negative for *MECP2* mutations, a hypothesis that should be investigated in the near future.

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