European Journal of Human Genetics (2013) 21, 1457–1461
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www.nature.com/ejhg

SHORT REPORT

Extended spectrum of *MBD5* mutations in neurodevelopmental disorders

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Intellectual disability (ID) is a clinical sign reflecting diverse neurodevelopmental disorders that are genetically and phenotypically heterogeneous. Just recently, partial or complete deletion of methyl-CpG-binding domain 5 (*MBD5)* gene has been implicated as causative in the phenotype associated with 2q23.1 microdeletion syndrome. In the course of systematic whole-genome screening of individuals with unexplained ID by array-based comparative genomic hybridization, we identified *de novo* intragenic deletions of *MBD5* in three patients leading, as previously documented, to haploinsufficiency of *MBD5*. In addition, we described a patient with an unreported de novo *MBD5* intragenic duplication. Reverse transcriptase-PCR and sequencing analyses showed the presence of numerous aberrant transcripts leading to premature termination codon. To further elucidate the involvement of *MBD5* in ID, we sequenced ten coding, five non-coding exons and an evolutionary conserved region in intron 2, in a selected cohort of 78 subjects with a phenotype reminiscent of 2q23.1 microdeletion syndrome. Besides variants most often inherited from an healthy parent, we identified for the first time a *de novo* nonsense mutation associated with a much more damaging phenotype. Taken together, these results extend the mutation spectrum in *MBD5* gene and contribute to refine the associated phenotype of neurodevelopmental disorder.
European Journal of Human Genetics (2013) **21,** 1457–1461; doi:10.1038/ejhg.2013.22; published online 20 February 2013

Keywords: *MBD5*; nonsense mutation; intragenic duplication; intellectual disability

INTRODUCTION

Methyl-CpG-binding domain 5 (MBD5) protein (OMIM *611472) is a member of the MBD protein family in which MECP2 (OMIM *300005) is involved in Rett syndrome, a prototypical neurodevelopmental disorder. *MBD5* contains five non-coding exons at its 5′-end, followed by 10 coding exons. Two isoforms have been described,[1] the longer one contains 1494 amino acids and is encoded by exons 6–15, the second one contains 851 amino acids and is encoded by exons 6–9. Functional studies suggested that MBD5 is likely to contribute to the formation or function of heterochromatin.[1] Isoform 1 of *MBD5* is highly expressed in brain and testis and isoform 2 is highly expressed in oocytes, which suggest a possible role in cerebral functions and in epigenetic reprogramming after fertilization. Recently, deletions encompassing *MBD5*, as well as intragenic *MBD5* deletions have been identified in individuals with a phenotype of intellectual disability (ID), seizures, significant speech impairment, and behavioral problems.[2–8] In this study, we used pangenomic array-comparative genomic hybridization (array-CGH) and capillary sequencing of *MBD5* gene to investigate DNAs from patients with unexplained ID. We further extend the mutational spectrum of *MBD5* with damaging intragenic duplication and nonsense mutation associated with a clinical spectrum of neurodevelopmental disorder.

SUBJECTS AND METHODS

Ascertainment of the patients

Patients with an unexplained developmental delay/ID as isolated symptom or in association with behavioral problems took part in a clinical diagnostic testing for genomic imbalance using array-CGH, following initial testing for karyotype (results normal), thanks to the national array-CGH network funded by the French Ministry of Health. To further elucidate the involvement of *MBD5* point mutations, we collected a clinically defined cohort of 78 individuals with moderate to severe ID without a known genetic cause (genomic copy number variants larger than 200 kb were previously excluded) and with significant clinical overlap with 2q23.1 deletion syndrome, reminiscent of Angelman-like phenotype or Smith–Magenis-like syndrome. More specifically, we included patients with ID, severe speech impairment, seizures, behavioral problems and in particular with autistic-like features. Informed consents were available for all tested patients.

Array-CGH analysis

Microarray-CGH analysis was carried out using 44K or 105K-oligonucleotide array (Agilent Technologies, Santa Clara, CA, USA) as previously described.[9] The array was analyzed with the Agilent scanner and the Feature Extraction software (v9.5.3.1; Agilent Technologies). A graphical overview was obtained using the CGH analytics software (v3.5.14; Agilent Technologies).

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Received 16 July 2012; revised 27 December 2012; accepted 24 January 2013; published online 20 February 2013

Genomic quantitative PCR

Quantitative PCR (qPCR) was performed on genomic DNA, using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). We designed primer sets in *MBD5* gene (all primer sequences used in this study are available on request). qPCR was carried out as previously described.[9] The *RPPH1* gene was selected as the control amplicon. Validation experiments demonstrated that amplification efficiency of the control and all target amplicons were approximately equal. All samples were run in triplicate. The dosage of each amplicon relative to *RPPH1* and normalized to control male DNA was determined using the $2^{-\Delta\Delta Ct}$ method.

Genomic sequencing

MBD5 ten coding, five non-coding exons (NM_018328) and one evolutionary conserved region in intron 2 were PCR amplified using standard procedures (available on request). PCR products were then purified and subjected to sequencing using BigDye Terminator kit (Applied Biosystems).

mRNA isolation, reverse transcriptase-qPCR

Total RNAs were isolated from PaxGen blood RNA tubes using RNeasy mini kit (Qiagen, Hilden, Germany). Family samples were collected for patients A and B (mother, father and brother). Male and female controls were collected for patients C, D and E. RNA was reverse transcribed through the use of random primers (Superscript, Invitrogen, Life technologies, Paisley, UK). Reverse transcriptase quantitative real-time PCR (RT-qPCR) was performed on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). We designed primer sets within *MBD5* (available on request). RT-qPCR was carried out in a total volume of 20 μl containing 10 μl of SYBR Green Master Mix (Applied Biosystems), 0.4 mM of each primer and 5 μl of complementary DNA (cDNA). Thermal cycling conditions were 95 °C for 20 s, followed by 40 cycles with 95 °C for 3 s and 60 °C for 30 s. The *ESD* and *ABL1* genes were selected as control amplicons. Validation experiments demonstrated that amplification efficiency of control and all target amplicons were approximately equal. All samples were run in triplicate. The dosage of each amplicon relative to *ESD* and *ABL1* and normalized to control male cDNA was determined using the $2^{-\Delta\Delta Ct}$ method.

cDNA sequencing

Primers were selected in *MBD5* exons. RT-PCR products were electrophoresed on agarose gels, purified with NucleoSpin Extract II kit (Macherey–Nagel, SARL, Düren, Germany) and sequenced using BigDye Terminator kit (Applied Biosystems).

RESULTS

Clinical reports

The clinical characteristics of individuals with MBD5-specific disruption are summarized in Table 1.

Patients A and B are monozygotic twin sisters. The father and the two first siblings are healthy. The mother was treated for epilepsy but treatment was interrupted during pregnancies. They were born prematurely without fetal distress. *Z*-scores of birth weight and length were at −1, and head circumference was in the normal range. They were noted to have global developmental delay. Patient A sat independently at 16 months and walked at 2 years 6 months, patient B sat independently at 17 months and walked at 3 years. Both spoke only single words and presented with stereotypies and autistic features. A brain MRI was normal. At the age of 3 years 6 months, both heights were at −3 SD, whereas weights and head circumferences were in the normal range. There is a isolated nostril anteversion on facial examination (Figure 1a).

Patient C,a male proband, was born following an uncomplicated, full-term pregnancy. Parents were non-consanguineous and healthy. Family history is otherwise unremarkable. Neonatal adaptation was normal. Birth weight (3300 g), birth length (49 cm) and head circumference (37 cm) were within the normal range. He presented with hypospadias and developed multiple bronchiolitis. He had an inguinal hernia repaired. He was noted to have global developmental delay. He walked at the age of 22 months, and language milestones were delayed. At the age of 4 years, height was 99 cm (median), weight 15 kg (median) and head circumference 52 cm (+1 SD). He presented with stereotypies. No specific dysmorphic facial features were observed. He had fifth finger clinodactyly. Although his intelligence had not been formally evaluated, his ID was estimated to be mild to moderate.

Patient D is the only child of healthy non-consanguineous parents. Pregnancy was uneventful to the exception of hemorrhage related to partial placental detachment at 3 months of gestation. She was born at term with normal growth parameters. Initial developmental milestones were reported normal. She walked at 19 months. The first words were pronounced at 13 months. Between 24 and 30 months of age, regression of language skills occurred with concomitant regression of response to social overture. She gradually developed problematic behavior, with stereotyped movements of the arms, and periods of hyperactivity and attention deficit. She was seen at the neuropediatric department at 2 years and 4 months and at 3 years and 6 months. There was no motor deficit. Slight symptoms of cerebellar syndrome were noted with oral dyspraxia. She was also seen at the outpatient genetics clinic at 3 years and 11 months. Growth parameters were within the normal range and clinical examination showed a round face, nostril anteversion and down-turned corners of the mouth (Figure 1a).

Patient E is a 10-year-old boy first seen at the age of 14 months because of developmental delay. He is the second of three children of healthy unconsanguineous parents. Pregnancy was reported as normal. He was born at 40 weeks by cesarean section because of placenta praevia. Neonatal adaptation was normal. Birth weight (2560 g), birth length (48 cm) and head circumference (34.5 cm) were in the low normal range. Since the first days of life, parents reported feeding difficulties. The boy developed opisthotonos during the first months of life. Unmotivated laughter was also reported. When first seen at 14 months, sitting was unstable, hand movements were poor, and language was absent. Eye contact was reported as easy. Some jerky movements were described. Length, weight, and head circumference were at the 50th percentile. Craniofacial examination was not specific with slightly broad forehead. EEG and cerebral MRI were normal. A screen for metabolic abnormalities and methylation analysis for Angelman syndrome were normal. *UBE3A* gene analysis (Dr Moncla, Marseille) was normal. At the age of 2 years 7 months, there was still no verbal language. Hypotonia was severe without walking. He presented with generalized tonico–clonic seizures at the age of 4 years. Treatment with valproate was initiated. At the age of 8 years, clonus of both legs were reported, which was associated with tongue and mouth clonus. EEG reported focal spikes and spike-wave complexes in the frontal and temporal left area, leading to the diagnosis of partial epilepsy. He was last seen at the age of 10 years 3 months. Height was at 147.5 cm (+2 SD), weight and head circumference were in the normal range. He stood independently for a short period of time but did not walk. There was no verbal language. His parents reported him as happy with very frequent smiles. Craniofacial examination showed unspecific hypotonic characteristics with long face, open mouth and slightly everted lower lip. Ear lobules were large (Figure 1a).

Molecular investigations

Array-CGH analysis demonstrated, according to UCSC build 36/hg18 (Figures 1b and c): (i) In patients A and B, an interstitial deletion at 2q23.1: arr 2q23.1(148 447 496–148 515 776) × 1, with a minimal size

Table 1 Clinical characteristics of the present patients in comparison with previously reported patients with *MBD5*-specific disruption (*Talkowski *et al.*[10])

		Present cases				
Patients	*Previous cases* % (frequency)*	*Patient A* *Female*	*Patient B* *Female*	*Patient C* *Male*	*Patient D* *Female*	*Patient E* *Male*
Current age (years)		$3^{6/12}$	$3^{6/12}$	4	4	$10^{3/12}$
Type and size of genomic anomaly	*MBD5*-specific disruption	Partial deletion 68 kb	Partial deletion 68 kb	Partial duplication 34 kb	Partial deletion 19 kb	Nucleotide substitution
Psychomotor retardation/ Intellectual disability	100% (14/14)	+	+	+	+	+
Language impairment	71.4% (5/7)	+	+	+	+	+
Autistic-like symptoms	100% (14/14)	+	+	−	+	+
Stereotypic repetitive behavior	60% (3/5)	+	+	+	+	−
Sleep disturbances	50% (3/6)	−	−	−	−	−
Short stature	40% (2/5)	+	+	−	−	−
Microcephaly	0% (0/5)	−	−	−	−	−
Seizures	85.7%(6/7)	−	−	−	−	+
Ataxia	0% (0/3)	−	−	−	+	−
Craniofacial features	66.6% (4/6)	+	+	−	+	+
Hand/foot anomalies	66.6% (4/6)	−	−	+	−	−
Urogenital abnormalities	0% (0/4)	−	−	+	−	−

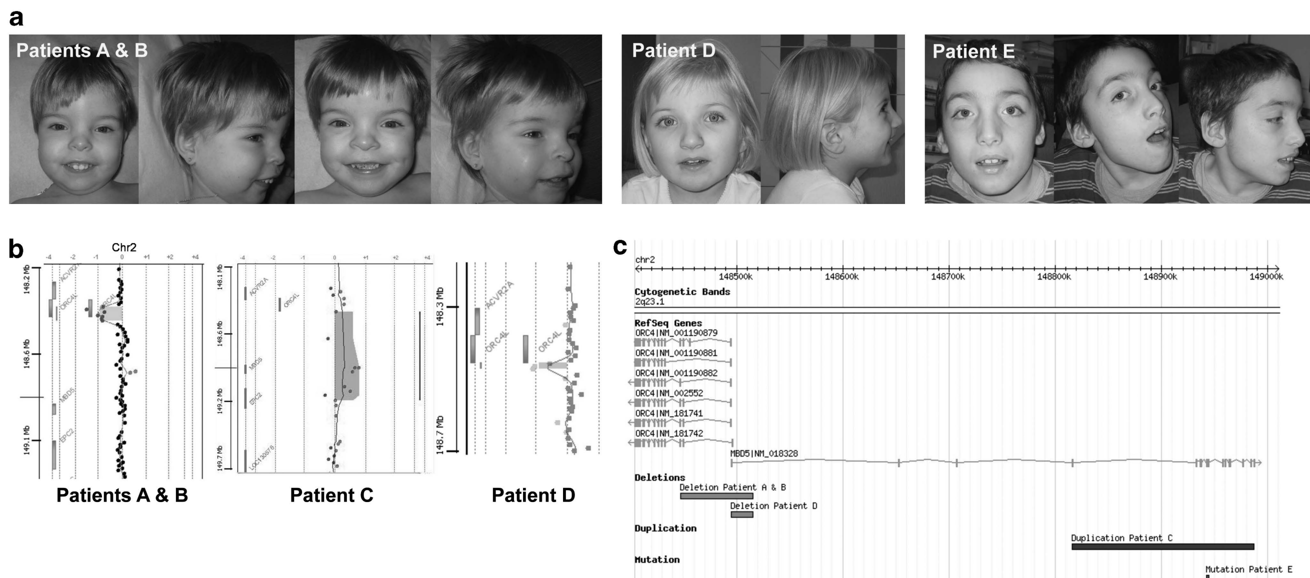

Figure 1 (**a**) Frontal and lateral view of patients A and B (3 years 6 months), patient D (3 years 11 months) and patient E (10 years), demonstrating a broad nasal bridge and hypoplastic nares. (**b**) 105K array-based CGH results showing the extent of *MBD5* intragenic deletion in patients A and B (7 probes) and for patient D (3 probes) and the extent of intragenic duplication for patient C (3 probes using 44K array-CGH) (**c**) Map of genomic alterations: deletions, duplication and nonsense mutation (snapshot of Database of Genomic Variants (http://projects.tcag.ca/variation/).

of 68 280 bp. The region includes the end of *ORC4* and the two first non-coding exons of *MBD5*. (ii) In patient C, an interstitial duplication at 2q23.1: arr 2q23.1(148 944 718–148 979 574) × 3 with a minimal size of 34 856 bp. This duplication affects only *MBD5*, the minimal duplicated region including four exons (5–8) and the maximal region including 11 exons (nc5–10). (iii) In patient D, an interstitial deletion at 2q23.1: arr 2q23.1(148 496 551–148 515 776) × 1 with a minimal size of 19 225 bp, including the end of *ORC4* and the two first non-coding exons of *MBD5*. This region had never been described as a copy number polymorphism in the database of genomic variants (http://projects.tcag.ca/variation/?source=hg18). Except for polymorphic regions, no copy number alterations were observed in other chromosomes. Using qPCR analysis on genomic DNA from patients A, B, D and their respective parents, we confirmed the biological relationships and revealed that genomic imbalances arose *de novo*. For patient C, parental DNAs were not available. However, qPCR on his genomic DNA allowed determining more precisely the extent of the duplication from non-coding exon 5 to coding exon 10. We used Sanger sequencing to screen *MBD5* for point mutations in the selected cohort of 78 individuals with ID. We identified a nonsense mutation (c.440C > G (p.Ser147*); NM_018328.3) within coding exon 4 in patient E (Figure 2a). Analysis of parental DNA confirmed the biological relationships and *de novo* occurrence of the mutation. In this series of patients, we also detected nine variants in protein-coding exons, not annotated in dbSNP (build 137), three intronic variants, three synonymous variants and three missense variants. In evolutionary conserved region and non-coding exons, five different variations were found. Detailed

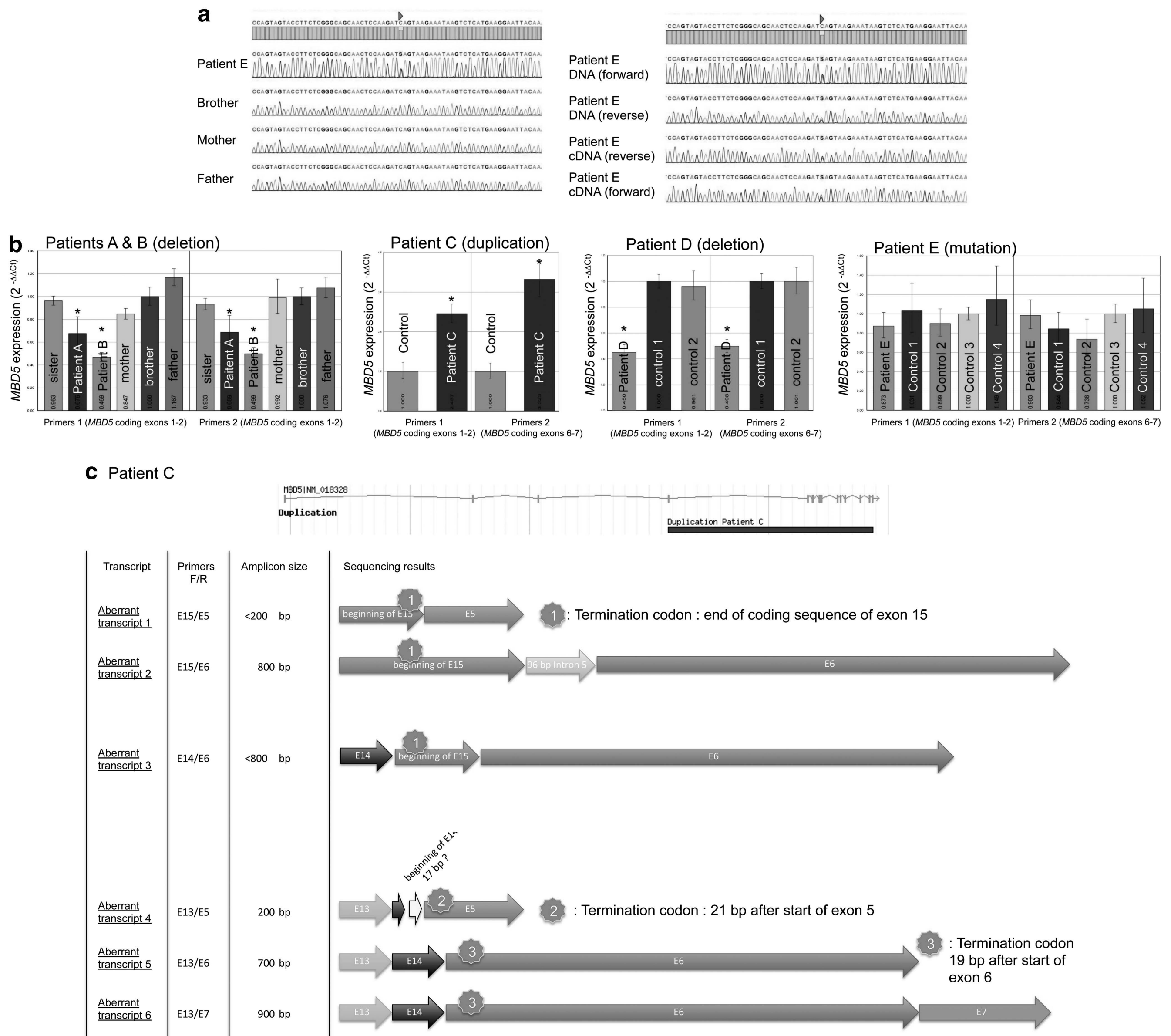

Figure 2 (**a**) Genomic sequencing results for patient E, his brother and parents showing (arrowhead) a *de novo* nonsense mutation in coding exon 4 (NM_018328.3:c.440C>G). Right panel: comparison of DNA and cDNA sequencing results in patient E showing that both normal and mutated alleles are expressed (arrowhead). (**b**) RT-qPCR results with primer set 1 (*MBD5*-coding exons 1–2) and primer set 2 (*MBD5*-coding exons 6–7): left and third panel, a decreased level of expression of MBD5 in patients A and B (compared with that healthy sister and parents), and in patient D (compared with that two controls), respectively, second panel, an increased level of expression for duplicated MBD5 exons in patient C (compared with that control), right panel, a normal level of expression of MBD5 in patient E (compared with that four controls). *indicates a significant difference. (**c**) Aberrant transcripts characterized by RT-PCR and sequencing analysis for patient C.

sequencing results are displayed in Supplementary Tables I and II. When parental material was available, we were able to show transmission from a healthy parent in all cases. RT-qPCR analysis showed (Figure 2b): (i) a notable reduction of *MBD5* expression for both sisters A and B and for patient D, (ii) a significantly increased level for duplicated MBD5 exons in patient C, and (iii) a normal level of expression for patient E. RT-PCR analysis in patient C, with forward primers in coding exons 8, 9 and 10 and reverse primer in exon nc5, coding exons 1 and 2 of *MBD5*, amplified different aberrant transcripts. Sequencing analysis of these fragments (Figure 2c) showed that all aberrant transcripts led to premature termination codon. For patient E, RT-PCR and sequencing analysis of exon 4 showed that both normal and mutated alleles were expressed (Figure 2a).

DISCUSSION

Recently, Talkowski *et al*[10] suggested a mixed model of deleterious, fully penetrant *MBD5* deletions causing a neurodevelopmental disorder associated with features of 2q23.1 microdeletion syndrome, and reduced penetrance missense variants that significantly increase risk for autism spectrum disorder. In our work, we identified five patients with *de novo MBD5*-specific disruption (for patient C, we are aware that parental DNA was not available to confirm *de novo* occurrence of the intragenic duplication) with clinical characteristics similar to previously reported patients[10] (Table 1), mainly with psychomotor retardation/ID, language impairment, and autistic-like symptoms. For patients A, B, C and D the phenotype is overlapping, less specific than patients with 2q23.1 microdeletion, which includes

more frequently microcephaly, small hands and feet, short stature, and broad-based ataxic gait. Developmental delay/ID is isolated in patient C, and associated with behavioral problems in patients A, B and D. Seizures were not observed in these four patients, at this time in development. At the opposite, the phenotype of patient E is much more damaging without walking and verbal speech at the age of 10 years.

Three patients (A, B and D) had a deletion including the last exons of *ORC4* and only the two first untranslated exons of the brain-expressed isoform 1 of *MBD5*. A similar deletion has been reported.[5,10] Expression level of *MBD5* mRNA in patients A and B was significantly reduced in comparison to their non-deleted parents, sister and brother. This result proves that heterozygous deletion of the two first non-coding exons of *MBD5* isoform 1 specifically leads to extinction of its expression on deleted allele. Interestingly, two novel *MBD5* genetic alteration types were identified, an intragenic duplication and a nonsense mutation. Patient C intragenic duplication affects non-coding exon 5 to coding exon 10 of *MBD5*. Transcriptional studies showed the presence of six aberrant transcripts, and sequencing analysis showed in each of these transcripts a premature termination codon (at the end of the coding sequence of exon 15, 21 bp after the start of exon 5, 19 bp after the start of exon 6) in favor of a modified MBD5 protein with putative altered function. Patient E *de novo* nonsense mutation leads to premature termination codon in *MBD5* gene, and is predicted to result in a truncated protein that lacks the Proline-rich domain in addition to the putative nuclear localization signal. This mutation was not reported in the 1000 Genomes project (http://browser.1000genomes.org/) or in dbSNP (builds 137). RT-PCR analysis showed a normal level of expression of *MBD5*, suggesting that RNA decay did not occur. Notably, *MBD5* transcripts sequencing showed *in vivo* expression of both normal and mutated transcripts. Translation of this mutated transcript might lead to a truncated protein with a dominant negative effect or this aberrant protein with the lack of the putative nuclear localization signal might impair the protein function. Complementary functional studies will help to appreciate the pathogenicity of these mutations. This fully penetrant mutation represents 1.2% (1/78) of our selected cohort. Interestingly, a *MBD5* frameshift mutation (c.150del (p.Thr52His*fs**31); NM_018328.4), resulting in a premature stop codon has been reported in a patient with Kleefstra syndrome phenotypic spectrum.[11] This frameshift mutation, predicted to be deleterious, is in favor of the implication of MBD5 mutations in an extended spectrum of neurodevelopmental disorders. Finally, regarding *MBD5* point mutations, missense variants have been reported,[2,10] mainly inherited from a healthy parent. We also identified in our selected cohort of patients (Supplementary Table I) previously reported missense variants. More specifically, for two patients (33 and 64) we detected the variants p.1048Thr > Ile and p.Ile752Val, respectively, each inherited from a healthy parent. As suggested by Talkowski *et al*,[10] these variants might participate as a potential risk factor for autism spectrum disorder. In conclusion, these findings confirm the involvement of *MBD5* mutations in neurodevelopmental disorders and extend the mutational spectrum of *MBD5*. Additional observations will be needed to establish fine genotype–phenotype correlations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank the patients and their family for their kind cooperation.
We thank the cytogenetics and molecular genetics staff at the Nancy Univesity Hospitals for their expert technical assistance. This study was supported by grants from the French Ministry of Health (DGOS) and the 'Fondation Jérôme Lejeune'.

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Supplementary Information accompanies the paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)