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ARTICLE

14q12 and severe Rett-like phenotypes: new clinical insights and physical mapping of *FOXG1*-regulatory elements

Lila Allou[1,15], Laetitia Lambert[1,2,3,15], Daniel Amsallem[4], Eric Bieth[5], Patrick Edery[6,7], Anne Destrée[8], François Rivier[9], David Amor[10], Elizabeth Thompson[11], Julian Nicholl[12], Michael Harbord[13], Christophe Nemos[1], Aline Saunier[14], Aissa Moustaïne[14], Adeline Vigouroux[5], Philippe Jonveaux[1,14] and Christophe Philippe*[,1,14]

The Forkhead box G1 (*FOXG1*) gene has been implicated in severe Rett-like phenotypes. It encodes the Forkhead box protein G1, a winged-helix transcriptional repressor critical for forebrain development. Recently, the core *FOXG1* syndrome was defined as postnatal microcephaly, severe mental retardation, absent language, dyskinesia, and dysgenesis of the corpus callosum. We present seven additional patients with a severe Rett-like neurodevelopment disorder associated with *de novo FOXG1* point mutations (two cases) or 14q12 deletions (five cases). We expand the mutational spectrum in patients with *FOXG1*-related encephalopathies and precise the core *FOXG1* syndrome phenotype. Dysgenesis of the corpus callosum and dyskinesia are not always present in *FOXG1*-mutated patients. We believe that the *FOXG1* gene should be considered in severely mentally retarded patients (no speech-language) with severe acquired microcephaly (−4 to −6 SD) and few clinical features suggestive of Rett syndrome. Interestingly enough, three 14q12 deletions that do not include the *FOXG1* gene are associated with phenotypes very reminiscent to that of *FOXG1*-mutation-positive patients. We physically mapped a putative long-range *FOXG1*-regulatory element in a 0.43 Mb DNA segment encompassing the *PRKD1* locus. In fibroblast cells, a *cis*-acting regulatory sequence located more than 0.6 Mb away from *FOXG1* acts as a silencer at the transcriptional level. These data are important for clinicians and for molecular biologists involved in the management of patients with severe encephalopathies compatible with a *FOXG1*-related phenotype.
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INTRODUCTION

Point mutations in the Forkhead box G1 (*FOXG1*) gene, encoding a brain-specific transcriptional repressor essential for the development of the telencephalon, were found to be responsible for congenital[1–7] or classical[2,5] Rett syndrome (RTT) in females and also in males.[5–7] Copy number variations (CNV) in 14q12 containing the *FOXG1* gene have been identified in both genders in patients suffering from neurodevelopmental disorders. Microdeletions in 14q12 were described in patients with early-onset Rett-like phenotypes associated with facial dysmorphic features.[8] In one case, a selective *FOXG1* deletion resulted in congenital Rett variant with minor facial dysmorphism (synophrys and pointed chin).[9] 14q12 microduplications encompassing the *FOXG1* locus also result in abnormal neurodevelopmental phenotype consisting of epilepsy (especially infantile spasms), mental retardation, and severe speech impairment.[10–13] Although the 14q12 CNVs contain additional genes, abnormal dosage of *FOXG1* is considered to be the best candidate to explain the neurodevelopmental disorders in these patients. Recently, Kortüm *et al*[6] reported an extensive clinical evaluation in a large series of patients heterozygous for a deleterious allele in *FOXG1*. The authors reported both point mutations and CNVs in 14q12, and proposed the designation of *FOXG1* syndrome for the clinical phenotype in patients with a *FOXG1* mutation. Patients with a chromosomal breakpoint[6,14] or deletions in 14q12[6,8] close but not disrupting the *FOXG1* gene have a phenotype similar to that of *FOXG1*-positive individuals. A position effect that causes altered expression of *FOXG1* has been proposed to explain the clinical features in these individuals.[6,14]

[1]EA 4368, Déficiences mentales et anomalies de structure du génome, Faculté de Médecine, Université de Lorraine, Vandoeuvre-les-Nancy, France; [2]Service de Médecine Infantile 1, Hôpital d'Enfants, Centre Hospitalier Régional et Universitaire, Rue du Morvan, Vandoeuvre-les-Nancy, France; [3]Unité de Génétique Clinique, Médecine Néonatale, Maternité Régionale Universitaire, Rue du Dr Heydenreich, Nancy, France; [4]Service de Neuropédiatrie, Hôpital St Jacques, Place St Jacques, Besançon, France; [5]Service de Génétique Médicale, Hôpital Purpan, Place du Dr Baylac, Toulouse, France; [6]Service de Cytogénétique Constitutionnelle, Hospices civils de Lyon, Groupement Hospitalier Est, BRON Cedex, France; [7]INSERM U102; CNRS UMR5292, University Lyon 1, Lyon Neuroscience Research Center, TIGER team, Lyon, France; [8]Institut de Génétique Humaine, Charleroi, Belgique; [9]CHRU Montpellier, Neuropédiatrie CR Maladies Neuromusculaires, Montpellier, France; [10]Murdoch Children's Research Institute, Royal Children's Hospital, Melbourne, Australia; [11]SA Clinical Genetics Service, SA Pathology, Women's and Children's Hospital, North Adelaide, SA, Australia; [12]Department of Cytogenetics, SA Pathology, Women's and Children's Hospital, North Adelaide, South Australia; [13]Department of Paediatric, Flinders Medical Centre, Bedford Park, South Australia; [14]Laboratoire de génétique médicale, Centre Hospitalier Régional et Universitaire, Rue du Morvan, Vandoeuvre-les-Nancy, France
[15]These authors contributed equally to this work.
*Correspondence: Dr C Philippe, EA 4368 UHP Nancy Universite, Laboratoire de génétique médicale, Centre Hospitalier Universitaire de Nancy Brabois, Rue du Morvan, Vandoeuvre les Nancy F-54511, France. Tel: +33 3 83 15 76 04; Fax: +33 3 83 15 37 72; E-mail: c.philippe@chu-nancy.fr
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Here, we described seven new deleterious alleles 14q12 consisting of two point mutations in *FOXG1*, three CNVs encompassing *FOXG1*, and two deletions distal but close to the *FOXG1* locus. The patients (four girls and three boys) presented with severe RTT-like neurodevelopment disorders. Using cultured fibroblasts, we determined the impact of three 14q12 CNVs on the expression of *FOXG1*. We confirmed and precisely mapped *cis*-acting regulatory elements distal to *FOXG1* that may act at the transcriptional level.

PATIENTS AND METHODS

Patients and phenotype definitions

In this study, we included individuals with Rett and severe Rett-like phenotypes who were recruited at different clinical genetic centres through France, Australia, and Belgium. The molecular and cytogenetic studies were performed at the molecular laboratory of the public hospital in Nancy, France. We tested typical RTT and congenital or early-onset RTT when the normal perinatal period was absent or shorter than 6 months. For almost all patients, mutations in *MECP2* and *CDKL5* genes could not be detected in these patients. We also included patients with a severe mental retardation associated with epilepsy, these individuals were negative for *MEF2C*. Total genomic DNA was extracted from peripheral blood using the Nucleon BACC genomic DNA extraction kit (GE Healthcare, Velizy-Villa Coubley, France). Biological samples from patients and clinical data were obtained after informed consent at all participating institutions.

Mutation screening of *FOXG1* by direct sequencing of PCR products

We analysed the entire coding sequence by direct sequencing of PCR products. Exon 1 of the *FOXG1* gene was PCR amplified (primer sequences available upon request). Primers were modified by the addition of either M13F (5′-tgtaaaacgacggccagt-3′) or M13R (5′-caggaaacagtcatgacc-3′) sequences at their 5′-end. The coding sequence was screened by direct DNA sequencing with M13F and M13R primers as described earlier.[15] Sequences were automatically analysed with the Seqscape 2.5 software (Applied Biosystems, Foster City, CA, USA). Sequence variants are numbered starting from the first base of the ATG codon, numbering based on reference sequence NM_005249.3. Naming of variants with the Alamut 2.02 software (Interactive Biosoftware, Courtaboeuf, France) follows the Human Genome Variation Society nomenclature.

Screening for large rearrangements of *FOXG1* by qPCR

Detection of large rearrangements of the *FOXG1* gene was performed by qPCR (quantitative PCR) for exon 1 (two amplicons) and exon 5 (one amplicon) (primer sequences available upon request). qPCR on genomic DNA was performed as described previously,[16] using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems).

Array CGH

The 14q12 deletion in patient 3 was identified by screening 18 patients with the Agilent kit 105A (Agilent Technologies, Santa Clara, CA, USA). This work was supported by a grant (CPRC 04.9582, 2009) from the Centre Hospitalier Universitaire de Nancy. The array-comparative genomic hybridisation (CGHa) analysis was performed as described previously.[16] The array was analysed with an Agilent scanner and the Feature Extraction software (v10.7.3.1, Essone, Massy, France). A graphical overview was obtained using CGH analytics software (v4.0.76, Essone). Validation of CNVs identified by CGHa was performed by qPCR. For each CNV, we tested three primer sets located in the chromosomal region of interest to establish the *de novo*/inherited feature of the chromosomal imbalance.

Cell culture

Fibroblast cells were maintained in Dulbecco's Modified Eagle's Medium (Gibco-Invitrogen, Illkirch, France) supplemented with 10% fetal bovine serum and antibiotics at 37 °C, 5% CO_2 under humidified atmosphere.

Analysis of the *FOXG1* transcript by RT-qPCR

Total RNAs from fibroblast cells (patients 3, 6, 7, and 8) were extracted with the TRIzol reagent (Invitrogen, Courtaboeuf, France). For patient 5, total RNAs were extracted from saliva with the Oragene•RNA Self-Collection Kit (DNA Genotek, Kanata, ON, Canada). RNA quality (RNA Integrity Number >9) was assessed with the Agilent 2100 Bioanalyzer. Before RT-PCR, RNAs were treated with DNase I (Sigma, Saint Quentin Fallavier, France) at room temperature for 15 min, DNase I was inactivated at 70 °C for 10 min. RT-PCRs were performed with primers located in exon 1 of *FOXG1* with the QIAGEN (Courtaboeuf, France) OneStep kit. Reverse transcriptase quantitative real-time PCR (RT-qPCR) was performed on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) as described previously.[16] The dosage of the *FOXG1*-derived mRNAs was performed with one amplicon (primer sequences available upon request). The relative quantification was performed with the 2^{-DDCt} method relative to *ESD* cDNAs as described previously.[16] All samples were run in triplicate.

Analysis of the *FOXG1* transcript by western blot

Proteins were isolated from fibroblast cells (patients 3, 6, 7, and 8) with the ProteoJET Cytoplasmic and Nuclear Protein Extraction kit (Frementas, ZA Courtaboeuf, France), and the concentration was determined using bovine serum albumin as a standard (BCA Protein assay, Pierce Biotechnology, Rockford, IL, USA). Total lysates were boiled in SDS sample buffer, separated by SDS-PAGE, and blotted to polyvinylidene fluoride membrane (Biorad, Marne la Coquette, France). Filters were blocked in tris-buffered saline tween 20 (0.1%) (TBST) (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% tween 20) plus 5% dried milk, and incubated with primary antibodies for 16 h at 4 °C. The following primary antibodies were used: rabbit polyclonal anti-FOXG1 (1:1000, Abcam, Paris, France) and rabbit polyclonal anti-beta Actin (1:2000, Abcam). After washing three times with TBST, filters were incubated with peroxidase-conjugated secondary antibody (anti-rabbit IgG; 1:2000/1:5000; Abcam) for 1 h at room temperature. Detection was performed by enhanced chemiluminescence (ECL Plus, Amersham, GE Healthcare) using the ChemiDoc software (BioRad, Marnes-la-Coquette, France).

Use of online bioinformatics tools

For the identification of conserved non-coding elements, we used the ESPERR (evolutionary and sequence pattern extraction through reduced representations, http://genome.cse.ucsc.edu/) computational method and the DCODE ECR browser (http://ecrbrowser.dcode.org) with the human March 2006 (hg18) assembly. We selected highly conserved non coding elements (HCNE) with a vertical viewing range between (0.25–0.4 (ESPERR) or containing a minimum of 100 bases with at least 70% identity (ECR browser)).

Description of sequence variants

Sequences variant in the *FOXG1* gene are numbered starting from the first base of the ATG codon, numbering based on reference sequence NM_001453.2. For CNVs detected by CGHa, the distances from the 14p telomere are derived from the NCBI genome browser (www.ncbi.nlm.nih.gov/gap, build 36).

RESULTS

Identification of point mutations in *FOXG1* and CNVs in 14q12

In the French laboratory, the molecular screening of *FOXG1* was performed by sequencing analysis and qPCR in 80 patients with RTT or RTT-like encephalopathies. These patients were negative for *MECP2* and *CDKL5*. For 18 patients with typical RTTs (nine cases), Hanefeld variants (five cases), or congenital RTTs (four cases), we also looked for CNVs by CGHa. Patients 1, 3, 6, and 7 were initially tested negative for *MECP2*. Both *MECP2* and *CDKL5* were excluded in patient 2. CNVs were identified by CGHa as a first-tier diagnostic test for patients 4, 5, and 8. We identified two point mutations and two CNVs in 14q12 (patients 1, 2, 3, and 4, Figure 1a). The c.256dupC/c.256delC were identified in patients 1 and 2, respectively. These in/del mutations result in frameshifts (p.Gln86ProfsX35/p.Gln86ArgfsX106), theoretically the resulting proteins miss all

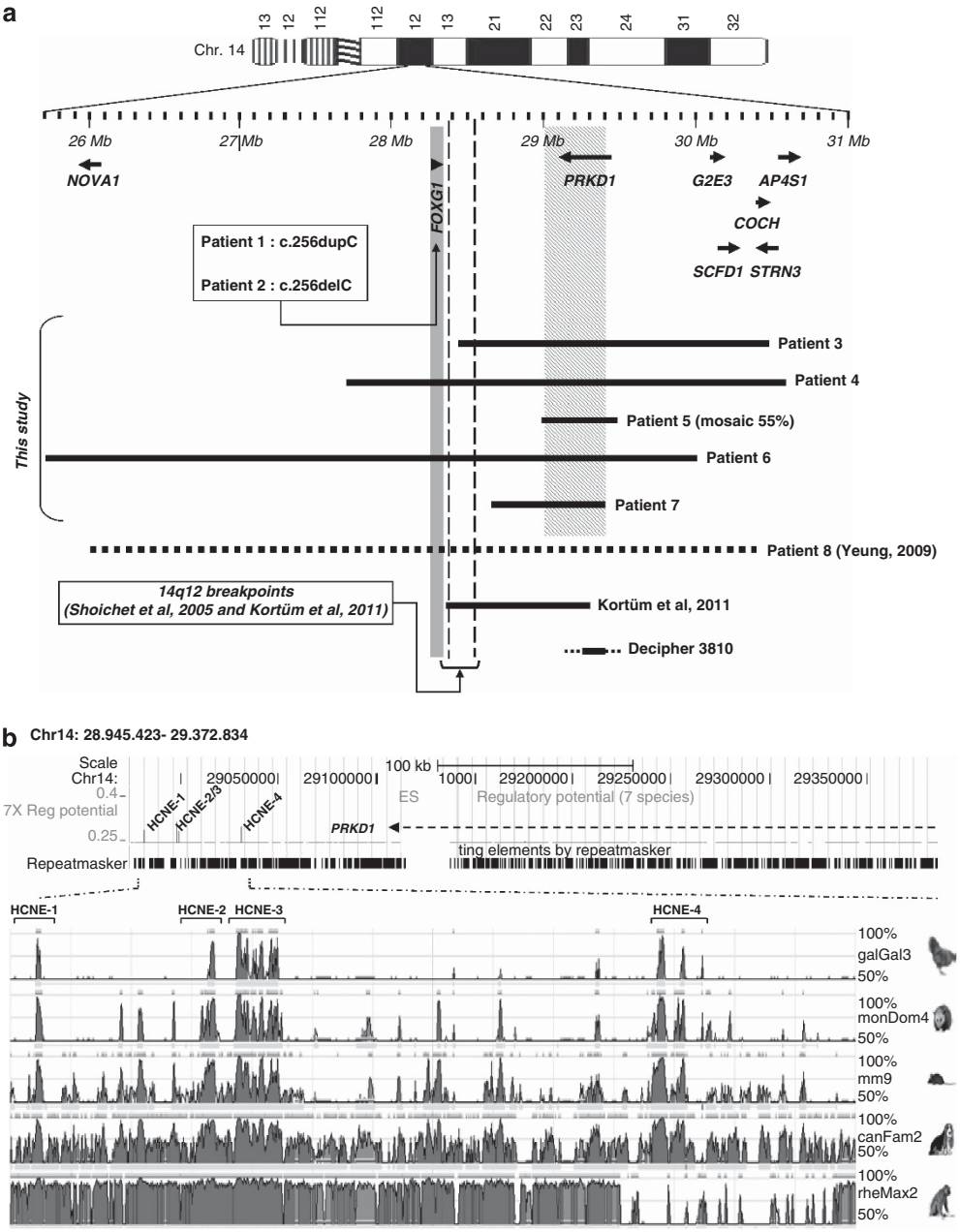

Figure 1 Physical mapping of *FOXG1*-regulatory elements in 14q12. (**a**) CNVs in 14q12 and *FOXG1*-related encephalopathies. We identified two point mutations in *FOXG1* (patients 1 and 2) and five CNVs in 14q12 encompassing or close to *FOXG1* (patients 3–7). The detailed view of the 14q12 region is derived from the NCBI genome browser (hg18, March 2006 assembly). Genes in 14q12 are represented by arrows (5′→3′ orientation). Deletions are depicted by horizontal black lines and duplication is depicted by an horizontal black dashed line. Vertical dashed black lines depict the location of 14q12 breakpoint in patients with a t(2;14). The minimal deleted region (28 945 423–29 372 834) is materialised by diagonal lines. (**b**) Bioinformatic predictions of putative *cis*-regulatory elements for the *FOXG1* gene. In the minimal deleted segment, the *FOXG1*/*PRKD1* intergenic region contains four HCNEs (HCNE1: 28 963 948-28 965 333; HCNE2: 28 981 071-28 982 265; HCNE3: 29 030 242-29 031 756; HCNE4: 29 066 922-29 068 642) identified by using the ESPRR computational method. The conservation throughout rhesus monkey, dog, mouse, opossum,and chicken of fragments >100 bp at 70% identity are indicated in red using the ECR browser (http://ecrbrowser.dcode.org).

functional domains of the FOXG1 transcription factor. The deletion in patient 3 is distal to the *FOXG1* locus. It extends on 2.1 Mb of DNA in 14q12 and is located <100 kb to *FOXG1*. The deletion in patient 3 was identified by CGHa, it includes 5 RefSeq genes: 14cen-*PRKD1-G2E3-SCFD1-COCH-STRN3*-14qtel. Patient 4 is heterozygous for a 3.2 Mb deletion that was identified by qPCR as part of targeted *FOXG1* screening. This deletion encompasses *FOXG1* as well as six other RefSeq genes (*PRKD1-G2E3-SCFD1-COCH-STRN3-AP4S1*). CNVs in patients 5–8 were identified by other laboratories. Patient 5 is a somatic mosaic for a 0.51 Mb deletion in 14q12 that removes part of the *PRKD1* (protein kinase D1) gene (exons 2–18). The neighbouring genes (*FOXG1* and *G2E3*) are not affected by this small deletion. FISH analysis on lymphocyte nuclei with BAC clones RP11-566C18 and RP11-181I14 revealed a 55% rate of mosaicism

(data not shown). Patient 6 (DECIPHER 251718) carries a 4.1 Mb deletion in 14q12 including *NOVA1*, *FOXG1*, and *PRKD1*. In patient 7, a CGHa analysis revealed a 0.62 Mb deletion distal to *FOXG1*, which encompasses part of the *PRKD1* gene (exons 2–18). In all seven cases, testing of parents revealed that the mutation occurred *de novo*. Sequencing analysis of the *FOXG1* coding region did not reveal any point mutation on the second allele in patients 3–7. Patient 8 was the first duplication in 14q12 including *FOXG1* described in the literature.[12] The microduplication extends on 4.45 Mb of DNA and involves six RefSeq genes: 14cen-*NOVA1-FOXG1-PRKD1-G2E3-SCFD1-COCH*-14qtel.

Clinical reports

Complete clinical descriptions for patients 1–7 are provided in the Supplementary Information. A summary is presented in Table 1. No common facial feature is remarkable in our cohort of seven *FOXG1*-mutated patients (Figure 2).

Quantification of the *FOXG1* expression

We established fibroblasts cultures from skin for patients 3, 6, and 7. For patient 8, we used fibroblasts from a cell line that was initially established in Dr Amor's laboratory. For patient 5, the parents did not allow a skin biopsy to be performed. The relative quantification of *FOXG1* transcripts was performed on total mRNAs extracted from cultured fibroblasts (patients 3, 6, 7, and 8) or from whole saliva (patient 5) (Figure 3a). In patients 3 and 7, the constitutional deletions in the vicinity of *FOXG1* are associated with a higher level of *FOXG1* mRNAs (increased level 28-fold and 2-fold, respectively). In cells from whole saliva, the *FOXG1* mRNAs level is also increased (twofold) in patient 5 who is a mosaic for the 14q12 deletion. Conversely, patient 8 with a 4.5 Mb duplication including *FOXG1* shows a 10-fold decreased of mRNAs level. The modifications at the RNA level were confirmed at the protein level (Figure 3b). The increase at protein level is also much higher for patient 3 ($10\times$) as compared with patient 7 ($2\times$). These results are in favour of a *cis*-acting silencer in the deletions/duplication overlapping region in 14q12 that impacts *FOXG1* expression. Surprisingly, the RNA/protein levels in patient 3 are much higher than in patients 5 and 7. There are several possible explanations for these variations. The *FOXG1* expression quantification has been performed on fibroblast cell lines at different passages that were established from patients at different age. The genetic background is different from one patient to another. More importantly, the deletion sizes are different in patient 3 (2.1 Mb), patient 5 (0.4 Mb), and patient 7 (0.62 Mb). The large deletion in patient 3 might remove additional sequences which coregulate the *FOXG1* expression. Moreover, the *FOXG1* gene is located in different local DNA sequence environments on the three deleted chromosomes. The chromatin conformation changes near the *FOXG1* locus could also explain the higher *FOXG1* expression in patient 3.

Physical mapping of long-range regulatory elements of *FOXG1*

We characterised three deletions in 14q12 that do not include *FOXG1* in patients with clinical conditions suggestive of *FOXG1*-associated phenotypes (Figure 1a, patients 3, 5, and 7). The minimal deleted area extends from the proximal boundary in patient 5 (28 945 423) to the distal boundary in patient 7 (29 372 834). Putative long-range regulatory elements might be located in this 0.43 Mb DNA segment located 600 kb distal to the *FOXG1* coding sequence. We compared the 430-kb human critical region with five other species by using two sequence alignment tools (ESPERR computational method and the ECR browser) (Figure 1b). Five highly conserved non-coding elements (HCNE) were identified in the intergenic region between *FOXG1* and *PRKD1*. These HCNEs were tested for gene enhancer activity *in vivo* in transgenic mice, these data are available by using the Vista Enhancer browser (http://enhancer.lbl.gov/). One out of five HCNEs act as an enhancer in the mouse forebrain, it was not considered as a candidate *FOXG1*-regulatory element. Indeed, according to our RT qPCR results the putative *cis*-regulatory sequence represses *FOXG1*.

DISCUSSION

We report on the clinical and molecular characterisation of seven new patients (four females and three males) with severe encephalopathies resulting from genetic abnormalities affecting *FOXG1* at the genomic or the transcriptional levels. Two frameshift mutations correspond to a 1 bp deletion or duplication in a poly-C stretch (c.250_256) in *FOXG1*. Another recurrent duplication (c.460dupG) was previously reported in three patients in a poly-G mononucleotide repeat.[5–6,17] The same c.256dupC frameshift has already been reported twice in patients with congenital RTT.[7,18] The c.256del/dup is the second mutational hot spot affecting a mononucleotide repeat in *FOXG1*. We also describe five new CNVs in 14q12 in two males and three females. In two cases, the deleted area encompasses *FOXG1*. Interestingly enough, three deletions close but distal to *FOXG1* involve all or part of *PRKD1*. In patient 7, the deletion affects only *PRKD1*.

Including this study, 26 point mutations, 25 CNVs, and 2 t(2;14) balanced translocations have been reported in patients with a *FOXG1*-related phenotype.[1–14,17–22] The male-to-female ratios is 7/26 (27%) for point mutations and 13/25 (52%) for CNVs. The preponderance of females in patients positive for a *FOXG1* point mutation is most probably due to the initial description of *FOXG1* deleterious alleles in females with congenital RTTs.[2,4] CNVs in 14q12 consist of deletions (14/25, 56%) and duplications (11/25, 44%). The vast majority of *FOXG1* duplications were found in males (9/11, 82%),[10–13] and are frequently associated with West syndrome (8/11, 72%)[10–13] or epilepsy with mental retardation and severe speech impairment.[11] Microcephaly is only present in 1 out of 11 patients with complete duplications of the *FOXG1* gene. However, the role of duplication of *FOXG1* in the pathogenesis of cognitive impairment and epilepsy is still a matter of debate.[23,24]

Combined with previous studies, our cohort suggests that the clinical phenotype associated with deleterious alleles affecting directly or indirectly *FOXG1* is heterogeneous (Table 1,[2–6]). In most articles, the *FOXG1*-related phenotype was reported as the congenital variant of RTT (MIM#613454). Recently, the *FOXG1*-related encephalopathy was described as a clinically recognisable phenotype designated as the *FOXG1* syndrome.[6] According to this study, the core syndrome phenotype consists of severe postnatal microcephaly, severe mental retardation, absent language, dyskinesia, and corpus callosum hypogenesis.[6] In addition, strabismus, protruding tongue, feeding difficulties, bruxism, and inexplicable laughing are often reported in patients heterozygous for a *FOXG1*-mutated allele. In three patients (2, 5, and 6), the phenotypes do correspond to the *FOXG1* syndrome with severe postnatal microcephaly (-4 SD to -5.5 SD), severe mental retardation without any language development, dyskinesia, and abnormal brain development. Patient 7 presented with a clinical presentation compatible with the *FOXG1* syndrome without corpus callosum dysgenesis. We, and others, previously described two females with a classical Rett linked to *FOXG1*.[2,5] Again in this series, patient 1 heterozygous for the c.256dupC has a phenotype very reminiscent of RTT with a normal psychomotor development for the first

Table 1 Molecular and clinical data for patients presented in this study

		P1 (female)	*P2 (male)*	*P3 (female)*	*P4 (female)*	*P5 (female)*	*P6 (male)*	*P7 (male)*
	Molecular analysis							
	- *FOXG1 point mutations*	c.256dup	c.256del	No	No	No	No	No
	- 14q12 CNV	No	No	2.1 Mb deletion distal to FOXG1	2.1 Mb deletion encompassing FOXG1	0.4 Mb deletion distal to FOXG1 – Somatic mosaicism 55% – *PRKD* is the only deleted gene	4.1 Mb deletion encompassing FOXG1	0.62 Mb deletion distal to FOXG1 – *PRKD* is the only deleted gene
	Clinical data							
	FOXG1 syndrome							
Core phenotype (Kortüm *et al*[6])	Severe postnatal microcephaly (−4/−6 SD)	Yes (−4 SD)	Yes (−5 SD)	Yes (−4 SD)	Yes (−4 SD)	Yes (−5.5 SD)	Yes (−4 SD)	Yes (−4 SD)
	Severe mental retardation	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	Absent language	Yes	Yes	Yes	Yes	Yes (few words)	Yes	Yes
	Dyskinesia	No	Yes	No	No	No	Yes	Yes
	Corpus callosum dysgenesis	No brain MRI	Yes (agenesis)	No	Yes (agenesis)	Yes (hypoplasia)	Yes (hypoplasia of the rostrum)	No
	Mild postnatal growth deficiency (−1SD/−2 SD)	Yes	No	No	No	Yes	No	Yes
	Rett syndrome							
Necessary criteria (Williamson and Christodoulou, 2006)[30]	Normal prenatal and perinatal history	Yes	No	Yes	Yes	No	Yes	Yes
	Nomal psychomotor development for the first 6 months	Yes	No	No	No	No	No	Yes
	Normal head circumference at birth	Yes (−1 SD)	Yes (−1 SD)	No (−2 SD)	Yes (0 SD)	No (− 4.5 SD)	Yes (−1 SD)	Yes (−1 SD)
	Postnatal decelaration of head growth	Yes	Yes	Yes	Yes	Yes	Yes	Yes
	Hand stereotypies	Yes	Yes (probable)	Yes	Yes	No	No	
	Social interaction	No	Poor eye contact	Poor eye contact	Good non-verbal contact	?	Yes	Progress in social reciprocity
	Speech	Never acquired	Never acquired	Never acquired	Never acquired	A few words at 30 months	Never acquired	Never acquired
	Hand skills	Never acquired	Never acquired	Regression	Never acquired	Acquired	Never acquired	Never acquired
	Locomotion	Never acquired	Never acquired	Never acquired	Never acquired	Acquired	Never acquired	Never acquired
Additional features	Hypotonia	Yes	Yes (severe)	Yes	Yes	Yes	No (dystonia)	Yes
	Strabismus	Yes	No	No	No	No	Yes, intermittent mainly after seizures	Yes
	Bruxism	Yes	No	No	Yes	No	No	No
	Breathing irregularities	No	No	No	No	No	No	No
	Epilepsy	No	Yes (1 year, refractory seizures)	Yes (7 months)	Yes (14 months)	No	Yes (severe, 8 months)	Yes (severe, 28 months)
	Poor sleep patterns	No		No	No	No	No	No
	Inexplicable episodes of crying/ laughing	Yes	Yes	Yes	No	No	No	Yes
	Gastro-oesophageal reflux	No	No	Yes	No	Yes	Yes	No
	Brain anomalies	Yes (reduced white matter in the frontal lobes)	Yes (poor myelination in the frontal lobes and enlarged ventricules)	Yes (reduced white matter in the frontal lobes)	Yes (reduced white matter in the frontal lobes)	Yes (poor myelination)	Yes (reduced white matter in the frontal lobes and hypoplasia of the cerebelar vermis)	Yes (poor myelination in frontal-subcortical brain)
	Tongue protruding movements	Yes	No	No	Yes	No	Yes	No
	Sialorrhea	Yes	No	No	No	No	Yes	No
	Orthopaedic deformations	Yes (scoliosis, flat feet)	No	Yes (scoliosis, decalcification)	Yes (scoliosis)	No	No	No
	Abnormal genitalia	No	No	No	No	No	No	Yes (micropenis, cryptorchidy)

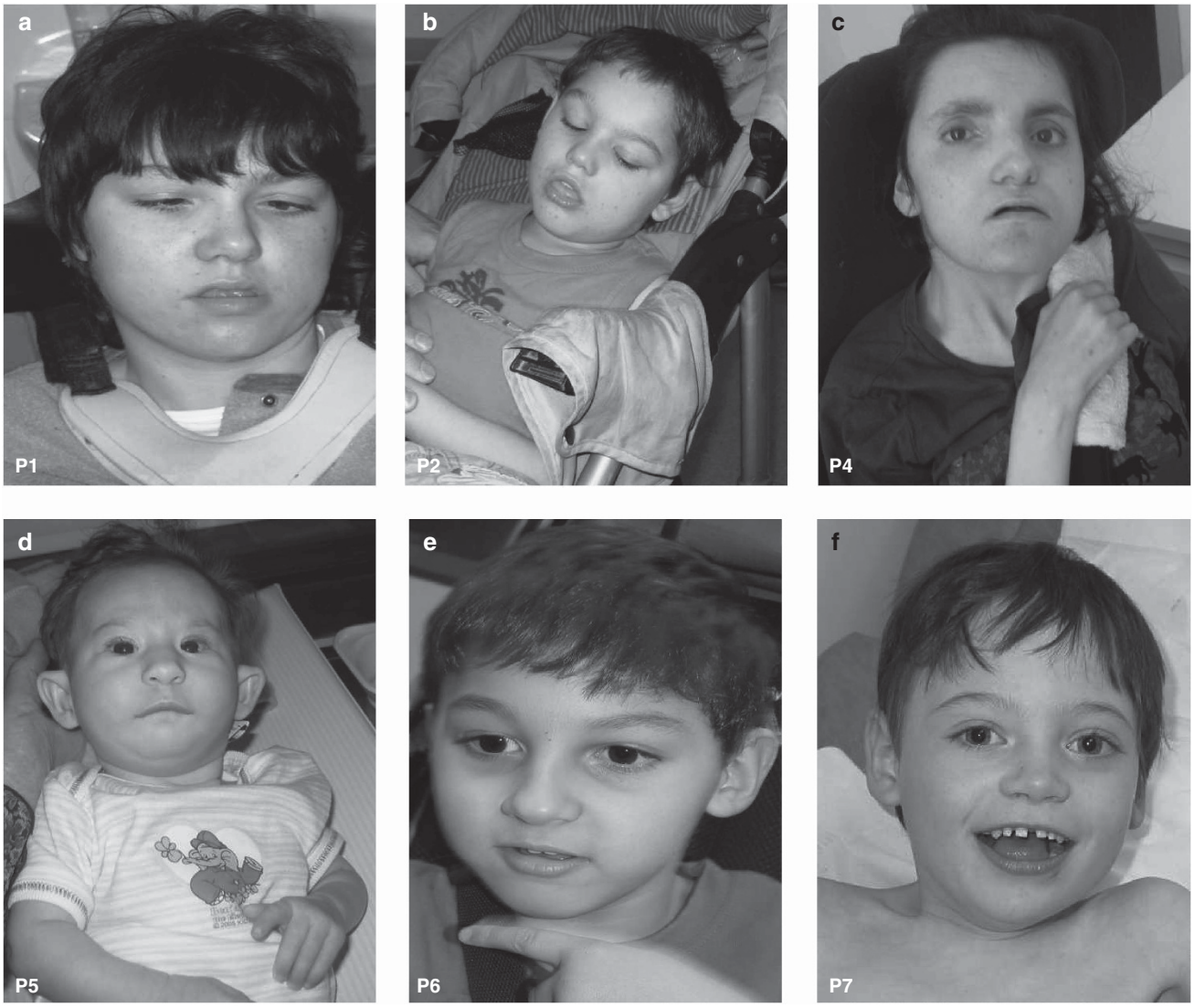

Figure 2 Clinical pictures of six out of seven patients presented in this study. Pictures (front view) of patients P1 (**a**), P2 (**b**), P4 (**c**), P5 (**d**), P6 (**e**), and P7 (**f**). No common facial feature is remarkable. Notice that patients are shown at very different ages.

6 months of life; however, she presented with a more severe acquired microcephaly than classically observed in RTT. One group hypothesised that deletion in the vicinity of *FOXG1* may be associated with normal corpus callosum.[6] Patients 3 and 7 presented with a normal corpus callosum. However, both patients 5 and DECIPHER 3810 with 14q12 deletions restricted to *PRKD1* showed hypoplasia of the colossal commissure. Additional genetic and/or environmental factors probably explain different phenotypic outcomes in patients with very similar *FOXG1* mutations.

Patients 3, 5, and 7 presented with small 14q12 deletions close but distal to *FOXG1* (Figure 1a). Patient 5 is a somatic mosaic, only half of her lymphocytes are heterozygous for the 14q12 deletion. All three patients have a phenotype reminiscent to that of *FOXG1*-mutated patients. The phenotype in patient DECIPHER 3810 with a deletion in the vicinity of *FOXG1* (http://decipher.sanger.ac.uk/) (Figure 1a) overlaps somehow with that of *FOXG1*-mutated patients. Notably, the *PRKD1* gene always lies within the deleted segment in all patients with a *FOXG1*-like phenotype associated with deletions distal to *FOXG1* (this study, DECIPHER 3810,[6,8]). In addition, two cases of t(2;14) balanced translocations with a 14q12 breakpoint distal but close to *FOXG1* have been reported in girls with phenotypes overlapping RTT with severe mental retardation and microcephaly[6,14] (Figure 1a). PRKD1 is a serine/threonine kinase that regulates a variety of cellular functions. Protein kinase D1 stimulates DNA synthesis and cell proliferation, and phosphorylates histone deacetylase (HDAC) 5 that is involved in control of chromatin structure and gene expression.[25,26] Protein kinase D1 also controls dendritic arborisation in hippocampal neurons.[27] The PRKD1 kinase phosphorylates class II HDACs. Phosphorylation-dependant nuclear export of HDAC4 and HDAC5 results in de-repression of downstream target genes.[25,28] Reduction of the PRKD1 kinase activity could explain the de-repression of *FOXG1* transcription in patients 3, 5, and 7 whereas an increased kinase activity could downregulate *FOXG1* expression in patient 8. To date, no clinical

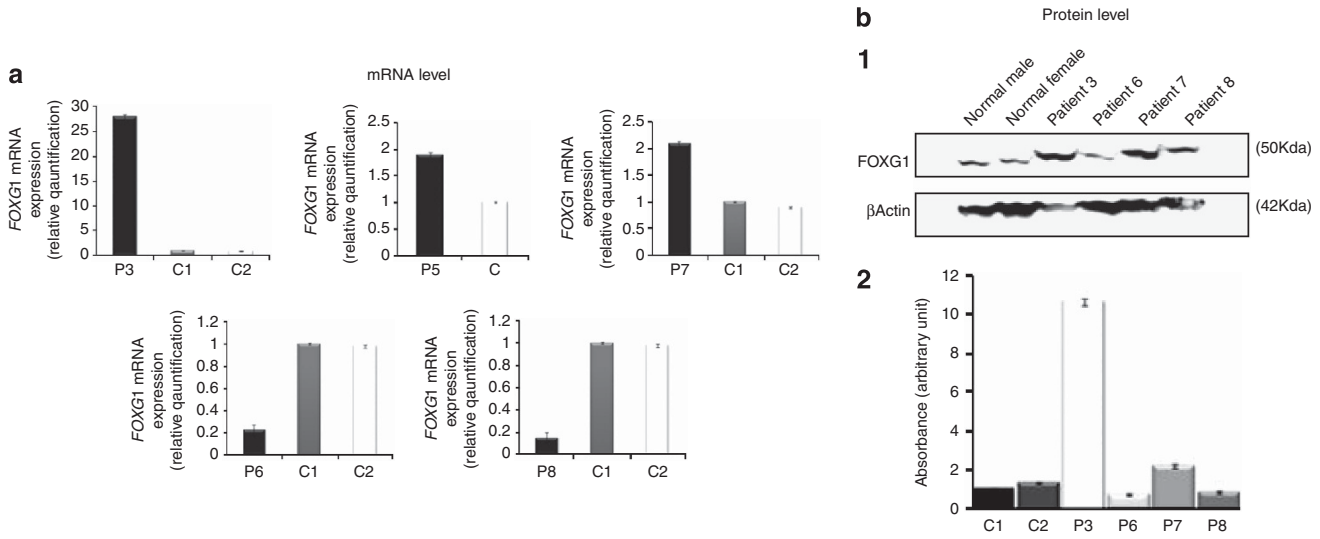

Figure 3 Quantification of the expression of *FOXG1* at the mRNA and protein level. (**a**) Quantification of *FOXG1* expression by RT-qPCR on total RNAs extracted from fibroblast cells (patients 3, 6, 7, and 8) or from whole saliva (patient 5). *FOXG1* mRNA level is increased in patients 3, 5, and 7 with a 14q12 deletion close to *FOXG1*. Conversely, the *FOXG1* expression is decreased in patient 8 (duplication including *FOXG1*) and in patient 6 (deletion including *FOXG1*). qPCRs have been performed in triplicate. (**b**) Quantification of *FOXG1* expression by western blot analysis on total proteins extracted from fibroblast cells (patients 3, 6, 7, and 8). The RT-qPCR results were confirmed by the quantification of the endogenous FOXG1 protein in patients 3, 6, 7, and 8 by immunoblotting with FOXG1 antibody. Densitometric quantification of the relative abundance of FOXG1 in each patient is presented in a bar graph. P: patient. C: control. β-actin was used as a standard in the quantification of the relative abundance of endogenous FOXG1.

entity has been associated with mutations or deletions in *PRKD1*. Although unlikely, mutations in *PRKD1* could be responsible for a clinical condition overlapping the *FOXG1*/congenital RTT. More than a hundred *FOXG1*-negative patients with a severe encephalopathy were screened retrospectively by qPCR directed towards exons 2, 10, and 16 of *PRKD1* (data not shown). For 11 patients with a clinical phenotype very evocative of the *FOXG1*-related encephalopathy, we also looked for point mutations in the coding sequence corresponding to the active site of the PRKD1 serine/threonine kinase. We did not find any large rearrangements or point mutation affecting *PRKD1* in these patients.

The *cis*-acting regulatory element hypothesis could explain the Rett-like phenotype in patients heterozygous for a 14q12 microdeletion close to *FOXG1*. Our results provide additional support for the existence of telomeric sequences regulating the expression of *FOXG1*.[6,14] Three additional CNVs allow us to narrow down the locus for a putative regulatory element in a 0.42-Mb DNA segment (28 945 423–29 372 834, hg18) corresponding to the smallest region of overlap (Figure 1a). Expression studies on cultured fibroblasts demonstrated that the expression of *FOXG1* is increased in patients 3, 5, and 7 and decreased in patient 8 with a microduplication encompassing the *FOXG1* locus (Figure 3). A regulatory element located more than 0.6 Mb away from the *FOXG1* coding sequence seems to act as a silencer at the transcriptional level. This regulation needs to be confirmed *in vivo* during the forebrain development. An unexpectedly high number of HCNEs correspond to functional *cis*-regulatory regions that influence gene transcription. Disruption of non-coding DNA sequences with regulation function of developmental genes is an emergent mutational mechanism in genetic diseases.[29] Two HCNEs that were proposed as candidate long-range *cis*-regulatory elements for *FOXG1*[6] do not map within the critical region as defined in this study. We selected four candidate highly conserved elements (Figure 1b) without enhancer activity as assessed in transgenic mice. We sequenced these highly conserved regions in 11 patients with a clinical phenotype very evocative of a *FOXG1*-related encephalopathy and did not find any deleterious mutations (data not shown). Additional patients and further studies will be necessary to track and characterise *FOXG1 cis*-regulatory sequences. Interestingly enough, a 88 kb duplication at 14q12 encompassing *FOXG1* and C14orf23 was found in a father and his son associated with a normal neurocognitive phenotype.[23] This duplication in 14q12 does not include the putative *cis*-regulatory elements mapped in the present study. We believe that this additionnal duplication in 14q12 strengthens the *FOXG1* long-range regulatory elements hypothesis. Importantly, the *FOXG1* molecular screening strategy should include a quantitative approach to look for deletions in the critical region harbouring putative *FOXG1 cis*-acting regulatory elements as defined in this study. We perform in a routine practice qPCRs with amplicons located in *PRKD1* (three amplicons) but also between *FOXG1* and *PRKD1* (four amplicons).

In conclusion, we expand the mutational spectrum in patients with *FOXG1*-related encephalopathies. We precise the core *FOXG1* syndrome phenotype;[6] dysgenesis of the corpus callosum and dyskinesia are not always present in *FOXG1*-mutated patients. A severe microcephaly (-4 SD to -6 SD) is always present in *FOXG1*-positive patients. Three additional deletions in the vicinity of *FOXG1* allow us to physically map a *FOXG1*-regulatory element in a 0.43-Mb DNA segment encompassing the *PRKD1* locus. In fibroblast cells, a *cis*-acting regulatory sequence located more than 0.6 Mb away from *FOXG1* acts as a silencer at the transcriptional level. These data are important for clinicians and for molecular biologists involved in the management of patients with severe encephalopathies compatible with a *FOXG1*-related phenotype.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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