Array-CGH Analysis Suggests Genetic Heterogeneity in Rhombencephalosynapsis

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Key Words

Array-CGH · Copy Number Variation · *NDE1* · Rhombencephalosynapsis

Abstract

Rhombencephalosynapsis is an uncommon, but increasingly recognized, cerebellar malformation defined as vermian agenesis with fusion of the hemispheres. The embryologic and genetic mechanisms involved are still unknown, and to date, no animal models are available. In the present study, we used Agilent oligonucleotide arrays in a large series of 57 affected patients to detect candidate genes. Four different unbalanced rearrangements were detected: a 16p11.2 deletion, a 14q12q21.2 deletion, an unbalanced translocation t(2p;10q), and a 16p13.11 microdeletion containing 2 candidate genes. These genes were further investigated by sequencing and in situ hybridization. This first microarray screening of a rhombencephalosynapsis series suggests that there may be heterogeneous genetic causes.

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Rhombencephalosynapsis (RS) is a rare congenital defect of the cerebellum. It is classically defined as vermian agenesis or hypogenesis with fusion of the cerebellar hemispheres. Since the first description by Obersteiner [1914], less than 200 cases have been reported. Magnetic resonance imaging (MRI) of a series of 3,000 consecutive paediatric brains led to its frequency being estimated at 0.13% [Sener, 2000]. However, the systematic use of MRI in the prenatal period suggests that the frequency of RS may be much higher: we have observed 40 foetal cases over 15 years [Pasquier et al., 2009]. Ishak et al. [2012] identified RS in almost 10% of patients with aqueductal stenosis, indicating that RS is more common than previously thought.

The severity of the clinical presentation is highly variable. In the prenatal period, RS is usually suggested by ventriculomegaly. Complete autopsy allows pure neurological phenotypes and those associated with extraneural anomalies to be distinguished from syndromic forms: Gomez-Lopez-Hernandez (GLH) syndrome (MIM 601853) [Lopez-Hernandez, 1982; Poretti et al., 2008]

and VACTERL-H syndrome (MIM 276950) [Michaud et al., 1982; Pasquier et al., 2009]. In foetal cases, isolated RS without fusion of the colliculi (also named mesencephalosynapsis) or aqueductal anomalies are never observed; various associated supratentorial abnormalities, such as agenesis of the corpus callosum, atresia of the third ventricle and holoprosencephaly, have been described [Pasquier et al., 2009; Mercier et al., 2011; Ishak et al., 2012]. In the postnatal period, there is impaired neurological function in most cases [Romanengo et al., 1997; Danon et al., 2000], although in some cases of RS cognitive functions are normal [Obersteiner, 1914; Bell et al., 2005; Poretti et al., 2009]. The neurological outcome is difficult to predict during pregnancy, but supratentorial [Sandalcioglu et al., 2006] and chromosomal anomalies [Lespinasse et al., 2004] are each always associated with poor prognosis. This has been confirmed by a large series of 42 postnatal cases [Ishak et al., 2012]. The imaging features that correlated with poor neurodevelopmental outcome were holoprosencephaly, severity of ventriculomegaly, aqueductal stenosis, fused colliculi, and abnormal temporal cortex.

RS is considered to be a sporadic condition and its aetiology remains poorly understood. Several factors have been suggested to be involved, including teratogenic agents like phencyclidine [Michaud et al., 1982; Sergi et al., 1997], chromosomal [Truwit et al., 1991; Lespinasse et al., 2004] and genetic [Yachnis, 2002] factors. Reviews of foetal cases led to the suggestion that RS may be the result of defects in genes regulating the formation of the roof plate and development of the midline cerebellar primordium at the junction of the mesencephalon and the first rhombomere between 28 and 42 days post-conception [Utsunomiya et al., 1998; Pasquier et al., 2009]. Genes implicated in early development have also been discussed as candidates, but no animal models have been described [Millonig et al., 2000]. It has not been possible to perform linkage analyses because familial cases are rare.

Only 2 different chromosomal abnormalities have previously been reported to be associated with RS. We therefore performed a genome-wide screening for submicroscopic anomalies in our RS cohort with no known karyotype alterations, using array comparative genomic hybridization (array-CGH). Four microrearrangements were identified, and 2 of them were demonstrated to be *de novo*. For the best candidate genes, we performed *in situ* hybridization to localize gene expression in the chick embryo model and screened for mutations.

Methods

Patients

We studied 57 cases (from 56 families) with normal karyotypes including 49 fetuses and 8 children or adults; these cases were identified and included through a collaborative study involving several French centres over the last 2 decades. We searched for consanguinity and recurrences as often as possible.

In all foetal cases, pregnancy was terminated either for hydrocephalus or RS discovered by ultrasound or MRI screening, according to the French law. These fetuses underwent a complete autopsy by standardized protocols. The pathological findings were reviewed systematically and have been reported previously [Pasquier et al., 2009].

The diagnosis of RS in live-born patients was supported by brain MRI screening performed for mental retardation or neurological symptoms.

Parents of all patients gave their informed consent.

Array-CGH

Blood samples, chorionic villi, amniotic fluid or foetal tissues were processed by the Molecular Genetic Laboratory of Rennes for microarray analysis.

Oligonucleotide array-CGH was performed after DNA extraction using the Agilent Human Genome CGH microarrays 44K (44 patients) and 180K (13 patients) (Agilent Technologies). Reference genomic DNAs from male and female controls were used to characterize copy number polymorphisms (CNPs). Microarrays were scanned using the Agilent scanner of the Biogenouest® Genomic Platform of Rennes. Images were analysed with Agilent Feature Extraction Software version 10.5.

Rearrangements identified by array-CGH were confirmed either by fluorescence *in situ* hybridization (FISH), multiplex ligation-dependent probe amplification (MLPA) or multiplex PCR liquid chromatography (MPLC).

Sequencing

The 5 coding exons of the *C16orf45* gene were amplified by PCR and analysed by direct sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the ABI Prism 3130 Genetic Analyzer on the whole cohort.

The 8 coding exons of the *NDE1* gene were amplified by PCR and analysed as well only on case 4.

Whole-Mount *in situ* Hybridization

To characterize the spatiotemporal expression of the candidate genes suspected after microarray analysis, we performed whole-mount *in situ* hybridization on chick embryos using antisense digoxigenin-labelled riboprobes as previously described [Chapman et al., 2002]. In order to clone the chick orthologs of the candidate genes, we used sequence-specific primers for each of the genes designed according to the chick reference sequences (www.ensembl.org). *NDE1* cDNA was obtained from the total RNA isolated from chicken brain (Zyagen Laboratories) by reverse transcription PCR using left (CAGAACCTCCTCCAAGCAAG) and right (GCATTGGGTGGCTAATGTCT) primers for *C16orf45* and left (TCCCTCAGTGTAGGGTTTGG) and right (GTGAAGGATG-GCTATTGGT) primers for *NDE1*. The amplified product was cloned into the PCRII TOPO vector (Invitrogen) and sequenced at the Institute of Genetics and Development of Rennes. For the

Table 1. Clinical and molecular features of the patients with pathogenic CNPs

Case	Sex	Child/foetus	Clinical features	Chromosomal rearrangement (size)	Inheritance	Breakpoints (hg19)
1	F	C	partial RS, growth retardation, developmental delay, dysmorphism	2pter duplication (10.5 Mb) 10qter deletion (9 Mb)	paternally balanced translocation	38,993–10,587,646 126,261,414–135,404,671
2	M	FOE	complete RS, hemivertebrae, costal anomalies	16p11.2 deletion (446 kb)	paternally inherited	29,673,754–30,119,912
3	F	FOE	complete RS, partial agenesis of corpus callosum	14q12q21.2 deletion (16 Mb)	de novo	27,867,357–44,582,530
4	M	C	partial RS, Gomez-Lopez-Hernandez syndrome	16p13.11 deletion (2.7 Mb)	de novo	15,256,486–18,012,602

C = Child; FOE = foetus.

construction of the sense probe, the plasmid was digested with *Bam*H1, while for the antisense probe the plasmid was digested using *Eco*RV. Riboprobes were transcribed from 1 µg of the linearized plasmids in the presence of DIG RNA labelling mix (Roche) and 20 U RNA polymerase (Roche), Sp6 for antisense and T7 for sense probes used as controls.

Results

Array-CGH

Thirty-four patients were male and 23 female (sex ratio 1.5). Two patients were siblings, and there was evidence of parental consanguinity in 2 other cases.

Chromosomal imbalances not described in the database of genomic variants (DGV) were detected in 4 (2 fetuses, 2 children) of the 57 patients (7%) (table 1), leading to the identification of new candidate loci. Additional copy number variants (CNVs) were discarded after interrogation of the DGV.

Case 1

Trisomy 2pter associated with monosomy 10qter was identified by multi-telomeric FISH in a 2-year-old girl with partial RS and growth and developmental delay associated with dysmorphic features. During the prenatal period, increased nuchal translucency, a slight hypoplastic cerebellum and intrauterine growth retardation had been observed. The rearrangement was found to be inherited from the father who carried a balanced subtelomeric translocation t(2p;10q) [Lespinasse et al., 2004]. The sizes of the rearrangements were determined from the 44K oligonucleotide Agilent array as 10.5 Mb for the 2pter duplication and 9 Mb for the 10qter deletion. Surprisingly, chromosome analysis using standard (450 bands) and high-resolution (800 bands) procedures showed a normal

karyotype probably because of the similar size of the rearranged telomeric regions.

Case 2

A heterozygous deletion of the region 16p11.2 was identified in a male foetus with a 44K microarray. The pregnancy was terminated medically at 19 weeks of gestation for hydrocephaly, cerebellar hypoplasia and a hemivertebra. The autopsy revealed complete RS, costo-vertebral abnormalities and dysmorphic features, as described previously [Pasquier et al., 2009]. This 446-kb deletion covering 27 genes was confirmed by MLPA (SALSA P343 kit, MRC Holland). The deletion was inherited from the phenotypically normal father.

Case 3

A de novo heterozygous deletion of 16 Mb in 14q12 encompassing more than 40 OMIM genes was detected in a female foetus (105K microarray). Hydrocephalus was diagnosed by the ultrasound scan during the second trimester. Morphological analysis of the foetus after medical termination of the pregnancy confirmed the typical cerebellar malformation associated with marked aqueductal stenosis and partial agenesis of the corpus callosum.

Case 4

A 2.7-Mb de novo deletion of the region 16p13.11 was identified by array-CGH using a 44K microarray in a 5-year-old boy who presented with learning difficulties. He had bilateral temporal alopecia and partial RS (fig. 1), strongly suggestive of GLH syndrome. This deletion covered 19 genes. Two of the deleted genes, *NDE1* and *C16orf45*, were selected as candidates and were further investigated by sequencing and in situ hybridization.

Fig. 1. Patient 4. **a** Bilateral alopecia. **b** Abnormal transverse folial running across the midline.

Fig. 2. Expression of chick *NDE1* at stage HH14 in whole embryos. **a** Lateral view. Left: in situ expression with the sense probe; right: in situ expression with the antisense probe. **b** Dorsal view. d = Diencephalon; mes = mesencephalon; met = metencephalon; mhb = midbrain-hindbrain boundary; ot = otic vesicle; r1–r5 = rhombomeres; t = telencephalon; v = optic vesicle. Bracket indicates the cerebellar territory in the hindbrain (metencephalon part).

Sequencing of *C16orf45* and *NDE1*

All 57 patients of the cohort were analysed for mutations in the 5 coding exons of *C16orf45*, including patient 4 with the deletion of one of the alleles. No mutation was found. Only 2 polymorphisms listed in the NCBI database were found in RS patients with the expected rate. The sequencing of the 8 coding exons of *NDE1* did not reveal any mutation of the remaining allele in case 4 (data not shown).

NDE1 and *C16orf45* mRNA in situ Hybridization Studies

We examined the expression pattern of *NDE1* and *C16orf45* in chick embryos during the characterization of the cerebellar territory in the hindbrain corresponding to Hamburger and Hamilton stage 14 (HH14). We observed no signal with the sense probes for *NDE1* and *C16orf45*, but with the antisense probe, we did observe strong specific expression of *NDE1* in the nervous system (fig. 2), whereas expression of *C16orf45* was ubiquitous (data not shown).

Discussion

We report the first large series of RS patients analyzed by array-CGH. Microrearrangements were detected in 7% of the RS cases. The microarray analysis did not identify recurrent rearrangements or deletions of regions encompassing genes known to be involved in embryogenesis of the cerebellum.

No patients with 10qter monosomy or 2pter trisomy have been reported in the literature to have RS [Courtens et al., 2006; Bonaglia et al., 2009]. The very large size of genomic regions involved did not allow us to highlight a particular candidate gene.

Microdeletions of a ~600-kb genomic region on chromosome 16p11.2, as in case 2, have been shown to be associated with a wide spectrum of neurobehavioral abnormalities, including developmental delay, autism, seizures [Shinawi et al., 2010], and obesity [Walters et al., 2010]. Congenital malformations have been reported in 30–50% of patients with CNVs of 16p11.2. The incidence of structural brain malformations is high among patients with the 16p11.2 deletion, but it is the first time that such a deletion is described in association with RS, suggesting either fortuitous association or low penetrance (the deletion is inherited from the healthy father – cerebral MRI not made) possibly through unmasking of recessive mutations.

Array-CGH analysis identified a chromosome 14 interstitial deletion of about 16 Mb in a foetus presenting partial agenesis of corpus callosum and complete RS associated with aqueductal stenosis. The deleted region encompasses the gene *FOXG1* which encodes a brain-specific transcriptional repressor that is essential for early development of the telencephalon. Mutations and deletions at this locus have been associated with congenital variant of Rett syndrome consisting of postnatal microcephaly, severe mental retardation, absent language, dyskinesia, and corpus callosum hypogenesis [Kortum et al., 2011], but no RS has been described.

The 16p13.11 deletion we report is particularly interesting because it was de novo; the patient shows features of the GLH syndrome whose aetiology remains unknown to date. Note that the 16p13.11 microdeletion syndrome is associated with neuropsychiatric disorders including schizophrenia [Ingason et al., 2011], developmental delay [Ullmann et al., 2007] and epilepsy [de Kovel et al., 2010]. Two candidate genes emerge from our study. The *NDE1* gene (nuclear distribution gene E nude homologue 1) seems to be a good candidate for the developmental delay of the patient. Indeed, whole-mount in situ hybridization for *NDE1* in chick embryos using antisense digoxigenin-labelled riboprobes showed strong and specific expression of *NDE1* in the nervous system, in particular at the mesencephalic-metencephalic junction during the development of the cerebellar territory in the hindbrain, corresponding to stage HH14 (fig. 2). This protein is essential for microtubule organization, mitosis and neuronal migration. Pawlisz et al. [2008] showed that *NDE1* interacts with *LIS1* to determine cerebral cortical size and lamination. Bakircioglu et al. [2011] recently described a human developmental disease involving extreme primary microcephaly with disordered cortical lamination (micro-lissencephaly) caused by biallelic *NDE1* mutations. The sequencing of the 8 coding exons of *NDE1* did not

reveal any mutation of the remaining allele in our case 4, excluding the possibility of its involvement following recessive inheritance. The second gene, *C16orf45*, is significantly expressed in the cerebellum according to the 'Gene Expression Omnibus' (GEO, NCBI) database and was expressed ubiquitously in the chick embryo at stage HH14 (data not shown). It encodes a protein that interacts with Enog, a protein with neurotrophic and neuro-protective properties [Hattori et al., 1995]. In this case, unmasking a recessive mutation by deletion of the second allele may have been the mechanism explaining the occurrence of GLH syndrome. However, this analysis failed to reveal deleterious mutations.

All cases with chromosomal anomalies presented with either supratentorial anomalies (hydrocephaly) or neurological disabilities. Therefore, it would be valuable to offer microarray screening during pregnancy when the diagnosis is suspected in order to predict the neurodevelopmental outcome as precisely as possible. Also, as RS is a multigenic disease with variable expressivity, inherited genetic anomalies may contribute to a RS genetic background, and thus these inherited deletions should be recorded.

The identification and characterisation of more genomic imbalances associated with RS will allow further delineation of minimal critical RS loci, which is the first step towards the identification of new RS genes.

Thus, total exome sequencing for cases of GLH and RS would be valuable and informative.

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