Periventricular heterotopia, mental retardation and epilepsy associated with 5q14.3-q15 deletion

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Abstract

Background: Periventricular heterotopia (PH) is an etiologically heterogeneous disorder characterized by nodules of neurons ectopically placed along the lateral ventricles. Most affected patients have seizures and their cognitive level varies from normal to severely impaired. At present, two genes have been identified to cause PH when mutated. Mutations in FLNA (Xq28) and ARFGEF2 (20q13) are responsible for X-linked bilateral PH and a rare autosomal recessive form of PH with microcephaly. Chromosomal rearrangements involving the 1p36, 5p15 and 7q11 regions have also been reported in association with PH but the genes implicated remain unknown. Fourteen additional distinct anatomicclinical PH syndromes have been described, but no genetic insights into their causes have been gleaned. Methods: We report the clinical and imaging features of three unrelated patients with epilepsy, mental retardation and bilateral PH in the walls of the temporal horns of the lateral ventricles, associated with a de novo deletion of the 5q14.3-15 region. We used microarray-based comparative genomic hybridization (CGH) to define the boundaries of the deletions. Results: The three patients shared a common deleted region spanning 5.8 Mb and containing 14 candidate genes. Conclusion: We identified a new syndrome featuring bilateral PH, mental retardation and epilepsy, mapping to chromosome 5q14.3-q15. This observation reinforces the extreme clinical and genetic heterogeneity of PH. Array CGH is a powerful diagnostic tool for characterizing causative chromosomal rearrangements of limited size, identifying potential candidate genes for, and improving genetic counseling in, malformations of cortical development.
Introduction

Periventricular heterotopia (PH) is a brain malformation caused by defective neuronal migration that leads to abnormal positioning of post mitotic neurons. Consequently, nodules of heterotopic gray matter accumulate along the walls of the lateral ventricles and can usually be detected using magnetic resonance imaging (MRI). There is a wide spectrum of anatomic and clinical presentations of PH, ranging from asymptomatic small unilateral or bilateral nodules to extensive agglomerates of heterotopia lining the lateral ventricles in patients with intractable epilepsy and intellectual disabilities. There is also a range of associated cerebral and systemic malformations. Two genes, \( FLNA \) and \( ARFGEF2 \), have been found to cause PH when mutated. \( FLNA \) maps to Xq28 and encodes for FILAMIN A, a very large (280 kD) cytoplasmic protein which binds to actin and a wide range of cytoplasmic signalling proteins involved in cell adhesion and migration. Almost 100% of families with X-linked bilateral PH and about 26% of sporadic patients harbor \( FLNA \) mutations. Heterozygous women have normal to borderline intelligence and epilepsy. Although a few living men with bilateral PH due to \( FLNA \) mutations have been reported, most male fetuses are not viable. Coagulopathy and cardiovascular abnormalities have been observed in some patients.

Mutations in \( ARFGEF2 \), in 20q13.1, are responsible for a very rare autosomal recessive form of PH associated with microcephaly and abnormal cortical folding. \( ARFGEF2 \) encodes a protein called BIG2 (or brefeldin-A inhibited GEF2 protein) involved in vesicular trafficking. PH has also been associated with deletions of the 1p36 or 7q11.23 regions and with duplications involving 5p15. To date, 14 additional distinct PH syndromes have been described but no genes identified.

Here we report three unrelated children, two boys and one girl, with severe mental retardation, epilepsy, and bilateral PH limited to the subependymal region of the temporal
horns, associated with a de novo deletion of the q14.3-15 region on chromosome 5. The
deletions range from 6.3 to 17Mb and share a common deleted region spanning 5.8 Mb.
Computational analysis of the critical region identified 14 candidate genes. This phenotype-
genotype association recognizes a new, genetically identifiable, syndrome.

Subjects and Methods

Patients
The study includes three patients from unrelated families from Italy, France and Australia.
These patients were identified in three centers where array CGH is being used to identify, or
accurately characterize, chromosomal rearrangements in individuals with developmental brain
abnormalities, mental retardation, and epilepsy. Clinical information, brain MRI and blood or
DNA samples were obtained after informed consent.

Single Nucleotide Polymorphism (SNP) Array CGH
Patient 3 had initially been studied using a Mapping 100K Set, which comprises two arrays
each with >50,000 SNPs, as previously described13.

Fluorescent In Situ Hybridization (FISH) analysis
In patients 1 and 3, an initial molecular cytogenetic study was executed using FISH with BAC
clones carried out on interphase or metaphase chromosome preparations by standard
methods14. For patient 1, whose unbalanced chromosomal translocation also included a
breakpoint at 1q31 (GenBank accession numbers for genes and BAC clones will be provided
on request) we used different prediction softwares in order to identify potential coding
sequences in this genomic region (see list provided as supplemental online material, E-Appendix 1).

Oligonucleotide Array CGH

Array CGH was performed in all three patients using a microarray kit (Human Genome CGH Microarray Kit 244A; Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer’s instructions. This kit uses an oligonucleotide-based platform that allows genome wide survey and molecular profiling of genomic aberrations with an average spacing of 6.5 kb. Labelling and hybridization were performed following the manufacturer’s protocols. Briefly, 500 ng of purified DNA from a patient and a control (Promega Corporation, Madison, Wisconsin, USA) were double-digested with RsaI and AluI for 2 hours at 7°C. After 20 minutes at 65°C, DNA of each digested sample was labeled by random priming (Invitrogen, Carlsbad, California, USA) for 2 hours using Cy5-dUTP for the patient DNA and Cy3-dUTP for the control DNA. Labeled products were column-purified and prepared according to the manufacturer’s protocol. After probe denaturation and pre-annealing with 50 mg of Cot-1 DNA, hybridization was performed at 65°C with rotation for 40 hours. After two washing steps, the arrays were analyzed with the Agilent scanner and Feature Extraction software (V.9.1.3.1). A graphical overview was obtained using CGH analytics software (V.3.4.27).

FLNA mutation analysis

The 47 exons covering the coding regions of FLNA (Entrez Gene, GeneID: 2316) and their respective intron-exon boundaries were amplified by PCR. Primer sequences and PCR conditions are available on request. PCR amplicons were purified using PCR clean-up kits (Sigma Aldrich, St. Louis, MO, USA and Agencourt, Beverly, MA, USA) and cycle
sequenced on both strands using the BigDye Terminator v.1.1 chemistry (Applied Biosystems, Foster City, CA, USA). The products were analyzed on 3100 ABI Prism DNA sequencer (Applied Biosystems, Foster City CA, USA).

**RT-PCR experiments**

Human tissue RNA samples were purchased from BD Biosciences Clontech (Palo Alto, CA). Human fetal brain RNA was isolated from normal fetal brain pooled from 21 spontaneously aborted male/female Caucasian fetuses ages 26-40 weeks. Human adult brain RNA was obtained from individuals who had died suddenly and unexpectedly with no evidence of disease. Whole brain RNA of 2 Caucasian men aged 47 and 55 years; cerebral cortex RNA was obtained from a pool of cerebral cortex tissues from 10 Caucasian men/women aged 20-68. Human lung, heart, skeletal muscle and lymphocyte RNA was obtained from healthy tissues of a pool of 20 individuals who had died between ages 15-68 years. Total lymphocyte RNA was extracted from human control lymphoblastoid cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from human RNA samples using Superscript II (Invitrogen, Carlsbad, CA) according to the manufacturer protocol. PCR conditions: 50 µl reaction containing 10x PCR buffer, 2 mM MgCl₂, 0.2 mM of each dNTP, 0.1 µM of each primer and 1 U of Taq polymerase (Invitrogen, Carlsbad, CA). polymerase (Applied Biosystems, Foster City, CA, USA). The reactions were denatured at 96°C for 4 min, followed by 25-30-35 cycles of 94°C for 35 sec, 56-62°C for 35 sec, and 72°C for 55 sec. Primer sequences are available on request.
Results

Subjects

(Clinical and imaging findings are summarized in E-Table 1)

**Patient 1** – This 7-year-old boy was born to unrelated healthy parents. At birth, a coloboma of the left iris was noted. Generalized hypotonia and developmental delay were reported in the first 2 years of life. At 3 years, clinical examination revealed minor cranial and facial dysmorphic features including a high forehead, hypertelorism, high arched eyebrows, mild downward slanting of the palpebral fissures, depressed nasal bridge, thick columella, and a flat long philtrum (E-Figure 1A). Left eye exotropia was also present. The boy walked independently at 5 years but remained markedly hypotonic and developed no language skills. At 1 age year, febrile seizures occurred. At 6 years, generalized tonic-clonic seizures appeared but were soon controlled with antiepileptic drugs. Electroencephalogram (EEG) showed normal background activity with no epileptiform discharges. Brain MRI revealed bilateral PH, involving the temporal and occipital horns (Figure 1A). Mild dilatation of the frontal horns was also present (Figure 1A).

Conventional chromosome analysis of peripheral blood lymphocytes showed a 46, XY, der(5) del(5) (q14;q21) t(1,5) (q31;q14) karyotype (Figure 2, A1) which had arisen de novo. FISH and array CGH were used to further characterize the rearrangement. First, we mapped the translocation breakpoint on chromosome 1 using BAC clones as FISH probes. BAC clones were chosen, on the Ensembl and the UCSC Genome databases, to cross the relevant region q31 on chromosome 1. FISH signals for BAC RP11-191J5 were observed on the derivative 1 and 5 chromosomes as well as the wild-type chromosome 1 (Figure 2, A2), localizing the 1q31 breakpoint to a position within this clone. Sequence analysis of BAC RP11-191J5 using
gene prediction softwares failed to identify any coding sequences in this genomic clone. In addition, there are no annotated genes near the breakpoint region as the most telomeric gene (RGS18) is located 597 kb from the breakpoint and the most centromeric gene (FAM5C) is located 910 kb from the breakpoint. Taken together, these data indicate that the 1q31 breakpoint does not interrupt or alter the function of any gene. Array CGH revealed a 5q interstitial deletion and localized the breakpoint to the 5q14.3-q15 region, spanning 17 Mb with 88,929,360-419 bp being the last oligomer deleted, and 105,929,496-555 bp the first oligomer present (genome assembly May 2004, hg17) (Figure 2 A3 and Figure 3). Mutation analysis of the FLNA gene gave negative results.

**Patient 2** - This 5-year-old girl was born to unrelated healthy parents. At birth, bilateral pes talus was observed; developmental delay was apparent in early infancy. At 9 months, infantile spasms appeared and proved resistant to antiepileptic medication. EEG showed poorly organized background activity and multifocal epileptiform discharges. At 3 years, epileptic spasms were still present. Clinical examination revealed severe developmental delay, absence of speech and minor dysmorphic features including a high forehead, frontal bossing, hypertelorism, anteverted nostrils, high arched eyebrows, depressed nasal bridge, thick columella, long philtrum, thin lips and micrognathia (E-Figure 1B). At age 5, cognitive development was severely impaired and no language skills had developed. Brain MRI revealed bilateral PH, involving the temporal and occipital horns (Figure 1, B1- B2). Standard chromosome analysis of peripheral blood lymphocytes showed a normal 46,XX karyotype. Array CGH revealed a 5q interstitial deletion and localized the breakpoint to the 5q14.3-q15 region, spanning 8.4 Mb with 87,086,298-357 bp being the last oligomer deleted, and 95,538,640-699 bp the first oligomer present (genome assembly May 2004, hg17) (Figures 2B and 3). The deletion occurred *de novo*. Mutation analysis of the FLNA gene gave negative
results.

**Patient 3** - This 5-year-old boy was born to non-consanguineous healthy parents. At birth, right postaxial polydactyly of his toes was noted. Early clinical evaluation revealed a triangular shaped head and poor truncal tone with variable tone of the limbs. From 8 months, episodes of unresponsiveness lasting 10-20 seconds occurred many times a day. At 18 months these episodes ceased but isolated myoclonic jerks appeared. Valproate treatment reduced the myoclonic seizures, which completely ceased by 3 years. Several EEG recordings showed bursts of multifocal and bilaterally synchronous epileptiform activity. The patient walked at 3 years; at 5 years, he was severely delayed and had no speech. Clinical examination revealed macrocephaly (>98th centile). Brain MRI revealed bilateral PH, involving the temporal and occipital horns (Figure 1, C1-C2). In addition, there were under rotated hippocampi, more severely on the right (Figure 1, C2) and irregular thickening and folding of the cortex in the posterior perisylvian regions, consistent with polymicrogyria (Figure 1, C3).

Molecular karyotyping for this patient was previously reported by Slater and collaborators. By conventional chromosome analysis of peripheral blood lymphocytes an abnormal 46,XY, del(5)(q14.2q15) karyotype was identified, which had occurred de novo. A high-density synthetic oligonucleotide array confirmed a 5q14.3-q15 interstitial deletion, spanning 6.3 Mb between 88,641,401 bp and 94,876,462 bp (genome assembly of May 2004, hg 17). Array CGH confirmed the 5q14.3-q15 deletion, spanning 6.3 Mb with 88,629,033-092 bp being the last oligomer deleted and 94,986,541-600 bp the first oligomer present (genome assembly May 2004, hg17) (Figures 2C and 3). FISH experiments, performed with BAC clones RP11-276J11 and RP11-626H3, confirmed the breakpoint location. Mutation analysis of the FLNA gene gave negative results.
Characterization of the PH critical region

To further define the critical region, we compared the size of the deletions between the three patients and found a common deleted region, spanning 5.8 Mb, distal to D5S2611 and proximal to D5S1943 (Figure 3B). Within this region, using the Ensembl and the UCSC Genome databases (E-appendix 1), we identified 14 annotated genes (Figure 3A). Semi-quantitative RT-PCR revealed that 13 of these genes are widely expressed in human fetal brain and adult specific brain regions (Table 1). The genomic region spanning from 93,790,962 and 94,067,957 bp contains a copy number variation (CNV) which consists of a copy number loss identified in the healthy population and containing the KIAA0825, C5orf36 and ANKRD32 genes (Database of Genomic Variants and Figure 3). This CNV was detected using a Whole Genome TilePath (WGTP) array that comprises 26,574 large-insert clones. However, neither SNPs arrays, nor other studies in which oligo aCGH/SNP arrays have been used, have subsequently confirmed this CNV.
Discussion

The three patients reported herein exhibited bilateral PH restricted to the temporal and occipital horns of the lateral ventricles, within the context of a clinical syndrome also featuring mental retardation, early onset epilepsy and minor dysmorphic facial features. Epilepsy had variable age of onset, seizure patterns and severity. This variability is often observed in PH and is probably related to the complexity of the epileptogenic networks which tend to involve the heterotopic neuronal aggregates as well as distant cortical areas, which can in turn be dysplastic. Cognitive delay was severe in all three patients and none had developed language skills. Such level of mental disability is much more severe than would be expected on the sole basis of the detectable heterotopia and likely indicates widespread cortical impairment. Polymicrogyria was clearly visible in patient 3 but no cortical abnormality was detected on MRI in the remaining two. Experimental evidence in rats, and neuropathological studies in humans, suggest that exogenous factors acting during pregnancy can cause PH. However, no definite anatomo-clinical patterns of PH have been definitely linked to exogenous factors. Conversely, considerable evidence has been gathered about the genetic causes and genetic heterogeneity of PH. Mutations of the FLNA gene are the main cause of ‘classical’ bilateral X-linked PH, which is the most commonly recognized phenotype. Rare FLNA mutations have been identified in patients with minor variants of PH such as unilateral PH, bilateral PH associated with Ehlers-Danlos syndrome and with severe constipation. In the three patients described here, clinical and brain MRI features differ from those due to FLNA mutations. In FLNA mutations, mental retardation is never severe and PH lines the walls of the lateral ventricles, especially the frontal horns and ventricular bodies, with limited or no extension to the temporal-occipital horns, and mega cisterna magna is often observed. Autosomal
recessive PH due to ARFGEF2 mutations also exhibits different characteristics in that it is associated with profound neurological impairment, early onset seizures, microcephaly and a simplified gyral pattern.

PH and mental retardation have also been described in association with known genetic syndromes or rare chromosomal rearrangements, mainly in isolated cases (E-Table 2). Several PH syndromes have also been reported for which the genetic bases are still unclear (E-Table 2)\(^2\,4\). Conversely, there is in vitro and in vivo functional evidence that loss of function of Mekk4 (a mitogen-activated protein kinase) causes PH in mice\(^23\), but no mutations of the MEKK4 gene have been identified in humans.

Hemizygous deletions of a specific chromosome are often associated with a contiguous gene deletion syndrome, in which the inactivation of several genes is involved in producing a complex phenotype. Molecular karyotyping in our patients established the size of interstitial deletions involving the 5q14.3-q15 region and defined a common 5.8 Mb deleted region; the clinical and imaging features appear to be sufficiently homogeneous to define a syndrome that can be assigned to the deleted region.

About 40 cases of constitutional interstitial deletion 5q have been reported, mostly before array CGH became available\(^24\), and only a few were studied with molecular markers, FISH or both. Overall, 20 patients have been reported whose deletions partially overlap the 5q14.3-q15 region\(^24\). Their phenotypic features include developmental delay and nonspecific dysmorphic facial features. No brain abnormalities were reported, although high resolution neuroimaging was not performed.

Unless the critical region is very small, it is usually difficult to identify whether a major gene exists for complex traits such as mental retardation and epilepsy, as the number of genes that
are expressed in the brain is high. PH is a marker of abnormal neuronal migration and might, as such, be linked to a specific gene, while mental retardation and epilepsy are likely to represent functional consequences of abnormal neuronal migration.

Of the 14 genes present in the common deleted 5.8 Mb region (Figure 3), based on expression patterns and functional data, 4 are good candidates for causing PH. The GPR98 gene (also known as VLGRI or MASS1) encodes for a member of the G-protein-coupled receptors family 2, which are probably involved in cell-cell adhesion. In situ hybridization studies on mouse embryos have shown that GPR98 is highly expressed in the central nervous system, particularly in the ventricular zone, and likely plays an important role in brain development.

Mutations in GPR98 have been found in a family with febrile seizures and have also been described in the Frings mouse, which exhibits audiogenic seizures. Therefore, GPR98 is a particularly attractive candidate gene for both developmental brain abnormalities and seizures. Centrin 3 (CETN3) encodes for one of the few identified centriolar proteins and is required for centriole duplication and inheritance. In vitro RNA interference experiments have shown that reduction of Centrin can result in cell division arrest and cytokinesis failure, which are interesting putative mechanisms for causing abnormal neuronal migration, considering that both FLNA and ARFGEF2 are implicated in controlling cell proliferation along the ventricular zone. MCTP1 (Multiple C2 and Transmembrane Region Protein 1) encodes for a new member of the C2 domains family proteins. Through their C2 domains, these proteins regulate the level of intracellular Ca2+ which plays an important role in controlling neuronal motility. COUPTF1 (chicken ovalbumin upstream promoter transcription factor 1; also known as NR2F1) encodes for an important regulator of neurogenesis, cellular differentiation and migration during embryonic development in several organisms, which appears to regulate the expression of two microtubule-associated proteins, MAP1B and MAP2. Abnormal migration or proliferation of neural progenitors induced by
dysregulation of the microtubule network is a plausible mechanism for causing PH. In addition, disruption of coup-tfl results in improper brain regionalization\textsuperscript{36} and abnormal cortical layering\textsuperscript{37} in mice; it is unknown, however, whether these mice have heterotopia.

Since the deletion involves four highly likely candidates, one cannot exclude that the syndrome may result from heterozygous loss of two or more of the four genes and that haploinsufficiency of only one may not be enough to produce the observed features.

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Figure Legends
Figure 1. Brain MRI of the three patients with 5q14.3-q15 deletion and periventricular heterotopia. **A.** Patient 1. T2 weighted axial section. Contiguous bilateral heterotopic nodules are present along the walls of the lateral ventricles in the temporal horns (arrows). **B1.** Patient 2. T2 weighted axial section. In the right temporal horn, contiguous heterotopic nodules are present along the ventricular wall (double arrow); an isolated nodule is visible on the left (single arrow). **B2.** Patient 2. T2 weighted coronal section. Contiguous nodules are lining the lateral ventricle on the right (double arrow). **C1.** Patient 3. T1 weighted coronal section, showing bilateral heterotopic nodules along the walls of the temporal horns (arrows). **C2.** Patient 3. T1 weighted coronal section taken 1 cm caudally to C2. On the right, heterotopia is still visible (arrow) and the hippocampal formation is rounded and under rotated. On the left, there is no heterotopia at this level and the hippocampal formation has maintained its horizontal axis. **C3.** Patient 3. T1 weighted coronal section taken 2.75 mm caudally to C2. There is marked infolding and thickening of the posterior perisylvian regions, more prominent on the right (two arrows).

Figure 2. Molecular karyotyping and array CGH in patients 1, 2 and 3 showing a 5q14.3-q15 deletion. **A1.** High resolution karyotype of patient 1 shows an unbalanced translocation between 1q31 and 5q14.3 (arrowhead), resulting in a deletion of the 5q14.3-q21.3 region. **A2.** FISH analysis on metaphase chromosomes using bacterial artificial chromosomes (BAC) RP11-191J5 (in red) reveals hybridization on the derivative 1 and 5 chromosomes, as well as the wild-type chromosome 1, localizing the 1q31 breakpoint within this BAC. **A3.** Oligonucleotide array CGH profile of patient 1 showing 5q14.3-q15 deletion. **B,C.** Oligonucleotide array CGH profiles of patients 2 and 3 showing 5q14.3-q15 deletions.

Figure 3. Delineation of the periventricular nodular heterotopia critical region
A. Physical and transcriptional map of the 5q14.3 to 5q21.3 region, which spans 20 Mb of genomic DNA. STS markers are depicted by black dots at the top of the figure. All known and new genes are placed exactly below the genomic region, which contains them; the arrows indicate the transcription sense. The copy number variant (CNV) present within the common deleted region is indicated by a black rectangle. B. Schematic representation of deletions identified in the three patients with PH. The solid lines below the map represent non-deleted genomic DNA and the dashed lines indicate the deletion identified in each patient. The shaded area delineates the common deleted region between the three patients.
**Table 1: Expression analysis of the genes localized in the PH critical region by semi-quantitative RT-PCR**

++ means that a signal is detectable after 25 PCR cycles; + means that a PCR fragment was detectable after 30 cycles of PCR; +/- means that a PCR fragment was detectable after 35 cycles of PCR; - means that we did not detect any expression after 35 cycles of PCR. G3PDH (Glyceraldehyde-3-phosphate dehydrogenase) gene was used as a positive control.  
*: POUSF2 expression is restricted to testis tissues.