Molecular characterization of 1q44 microdeletion in eleven patients reveals three candidate genes for intellectual disability and seizures

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ABSTRACT

Patients with a submicroscopic deletion at 1q43q44 present with intellectual disability (ID), microcephaly, craniofacial anomalies, seizures, limb anomalies and corpus callosum abnormalities. However, the precise relationship between most of deleted genes and the clinical features in these patients still remains unclear. We studied 11 unrelated patients with 1q44 microdeletion. We showed that the deletions occurred de novo in all patients for whom both parents’ DNA was available (10/11). All patients presented with moderate to severe ID, seizures and non-specific craniofacial anomalies. By oligoarray-based comparative genomic hybridization (aCGH) covering the 1q44 region at a high resolution, we obtained a critical deleted region containing two coding genes - HNRNPU and FAM36A - and one non-coding gene - NCRNA00201. All three genes were expressed in different normal human tissues, including in human brain, with highest expression levels in the cerebellum. Mutational screening of the HNRNPU and FAM36A genes in 191 patients with unexplained isolated ID did not reveal any deleterious mutations while the NCRNA00201 non-coding gene was not analyzed. Nine of the 11 patients did not present with microcephaly or corpus callosum abnormalities and carried a small deletion containing HNRNPU, FAM36A and NCRNA00201 but not AKT3 and ZNF238, two centromeric genes. These results suggest that HNRNPU, FAM36A and NCRNA00201 are not major genes for microcephaly and corpus callosum abnormalities but are good candidates for ID and seizures.
2 INTRODUCTION

Intellectual disability (ID) represents the most frequent cause of severe handicap in children and one of the main reasons for referral in clinical genetic practices. Causes of ID are extremely heterogeneous and can result from chromosomal rearrangements, monogenic disorders, and/or environmental factors. Despite clinical examination and extensive complementary investigations, no etiology is identified in up to 50% of the patients with moderate to severe ID [Chelly et al., 2006], hampering accurate genetic counseling and clinical follow-up. During these last years, the advent of high-resolution microarray techniques allowed for the detection of increasingly smaller rearrangements in patients with ID. The method has greatly facilitated deciphering chromosomal disorders, enabling better genotype – phenotype correlations and thus the identification of new genes responsible for ID.

A number of chromosomal regions scattered through the human genome are deleted in patients with ID, among them the 1q43q44 region. This deletion syndrome was first described by De Vries et al. [2001]. Patients present with ID, microcephaly, craniofacial anomalies, seizures, limb anomalies and corpus callosum abnormalities. However, the precise relationship between most of deleted genes and the clinical features in these patients still remains unclear. Three studies have each proposed a different smallest region of overlap (SRP) for corpus callosum abnormalities. A first critical deleted region described by Boland et al. [2007] was 1.25 Mb in size and contained two candidate genes: AKT3 and ZNF238. Next, van Bon et al. [2008] identified a second distinct critical region of 0.36 Mb in size, more telomeric than the first one, and containing four different candidate genes: Clorf100, ADSS, Clorf101 and PNAS-4. Caliebe et al. [2010] proposed a third interval of 0.44 Mb, which is more telomeric than the other two, and which contained the HNRNPU gene. Finally,
the combined data from two recent studies, a first one performed on 22 patients [Ballif et al., 2011] and a second one based on 7 patients [Nagamani et al., 2012] sharing 1q43q44 microdeletion, proposed three distinct SRO with different sizes implicated in corpus callosum abnormalities (75 kb in size, including ZNF238), microcephaly (133 kb in size, including AKT3) or seizures (100 kb in size, including HNRNPU, FAM36A and NCRNA00201 previously referred as C1ORF199). We focused our study on 11 unrelated patients with ID and seizures carrying a 1q44 interstitial microdeletion. We refined the SRO for ID and seizures to three genes and explored each of these three genes to highlight their potential role played in the phenotype.

Patients and Methods

Patients

We studied 11 unrelated patients (eight females and three males) with a 1q44 microdeletion. The non-specific craniofacial anomalies are presented in Figure 1 and clinical features are summarized in Table 1. The patients originated from Europe (France, Sweden, Finland, Monaco, The Netherlands and Germany) and Senegal. No consanguinity or familial genetic history was noted in the families. The pregnancies were uneventful.

All patients presented with moderate to severe ID, predominantly on verbal learning disabilities. Milestones were delayed in all patients: sitting unsupported ranged from 6 months to 3 years of age, walking unsupported from 2 to 5 years of age, and severe speech delay (6 patients aged from 4 years and 6 months to 17 years had no expressive speech and one patient had severe speech delay and phonetic disorders). Neurological examination revealed axial hypotonia in four cases. Five patients presented with stereotyped movements of the hands
with voluntary use of their hands conserved and were suspected for Rett syndrome. Two patients had sleep disturbances, of which one was treated with Melatonin with good results. Four patients had autistic features and/or attention deficit disorder.

Seizures were observed in all patients. The age of the first seizure ranged from 6 months to 2.5 years of age. Different types of seizures were observed: absences, generalized, tonic-clonic seizures and Lennox-Gastaut syndrome. Epilepsy required treatment in most patients, no pharmaco-resistant epilepsy was noted except for patient 3. Two patients developed status epilepticus history. Magnetic resonance imaging (MRI) revealed agenesis of corpus callosum in patient 2. The corpus callosum was normal in all other patients. MRI revealed other brain abnormalities: delayed myelinisation, generalized or subcortical atrophy, micropolygyria, moderate ventricular dilatation and moderate cerebellar hypoplasia. Only two patients (#1 and #2) among the eleven presented with microcephaly. Both patients carried the larger deletions including the AKT3 gene. This finding is consistent with other recent reports suggesting that AKT3 is a strong candidate gene for microcephaly [Ballif et al., 2011; Nagamani et al., 2012].

Six patients developed general obesity during childhood (BMI around +4 SD, obesity grade 2). Five patients had short stature (heights between -2.5 and -3 SD). Six patients had small hands and broad, short and/or small feet with small toes. Craniofacial anomalies were present in all patients, but did not lead to a characteristic facial dysmorphism. Hypertelorism, strabismus (4/9), bulbous nose (3/10), long and flat philtrum (5/10) and abnormal ears (4/10) were frequently observed.

Occasionally, some malformations were observed: unilateral renal agenesis, congenital heart defects (atrial septal defect and pulmonary stenosis), and some skeletal anomalies (a butterfly vertebrae, a scoliosis).
1 Cytogenetic and aCGH studies

Informed consent for genetic analyses was obtained from parents of the patients according to local ethical guidelines. Karyotyping based on R or G banding was performed using standard methods on metaphase spreads from peripheral blood of the patients. Genomic DNA was extracted from peripheral blood using standard protocols. Molecular karyotyping of the 11 patients was initially conducted using different array platforms according to manufacturers’ instructions. Subsequently, we used a custom targeted 60K Agilent array to fine map the breakpoints of the deletions with a median resolution of 240 bp. Custom arrays comprising 25,021 probes covered a 6 Mb in the 1q44 region including the HNRNPU, FAM36A and NCRNA00201 genes. Arrays were analyzed with a Agilent scanner and the Feature Extraction software (v. 10.5.1.1). Graphical overview was obtained using the Genomic Workbench software (v.5.0). Deletion breakpoints were mapped to the UCSC genome browser, hg19. A second independent method (fluorescence in situ hybridization (FISH) with different probes, qPCR or MLPA) was used to confirm the deletions and for parental inheritance in patients for whom DNA was available. Three individuals of Yoruba Nigerian origin from the HapMap Project were obtained from the Coriell Institute [IHMC, 2005] and were also analyzed using the custom 60K array.

20 mRNA expression studies

We performed expression analyses for HNRNPU, FAM36A and NCRNA00201 using total RNA extracted from different human tissues (primer sequences available upon request). RNAs were obtained from adult brain, heart, kidney, liver, cerebellum tissues and from fetal brain tissue (Clontech). Real time quantitative reverse transcription PCR (RT-qPCR) was
performed using the $\Delta\Delta$Ct method [Livak et al., 2001] to assess expression level of the three target genes - HNRNPU, FAM36A and NCRNA00201 – relative to the expression level of the $3\beta$-actin (ACTB) and cyclophilin E (PPIE) housekeeping genes. For a given target gene, the $4\Delta$Ct of each tissue was compared to the median of the $\Delta$Ct of the 6 tissues analyzed.

Mutation screening

The coding exons and the exon-intron boundaries of the HNRNPU and FAM36A genes were sequenced in 9/11 patients with a 1q44 deletion using the Sanger technology and run on ABI 3130 (primer sequences available upon request). A series of 191 patients with unexplained isolated ID were used to search for point mutations in HNRNPU and FAM36A. Standard karyotyping was normal in all 191 patients. In addition, molecular karyotyping performed with a 44K Agilent array was normal in 112/191 patients while the other patients were not analyzed. PCR amplifications followed by high-resolution melting method (HRM) were performed to screen HNRNPU (exons 2-14). PCR amplifications followed by Sanger sequencing were performed to screen the 5’ half of exon 1 of HNRNPU and the four exons of FAM36A. We failed to sequence the 3’ half of exon 1 of HNRNPU.

RESULTS

Cytogenetic and aCGH results

Following normal standard karyotyping, a 1q44 microdeletion was identified in 11 patients with moderate to severe ID, craniofacial anomalies and seizures using different high-resolution array platforms. No other pathogenic genomic imbalances were identified in the
patients. All deletions were confirmed by FISH, qPCR or MLPA. Parental analyses demonstrated de novo deletions in all families when both parents’ DNA was available. Using a custom targeted aCGH method, we showed that the sizes of the deletions were variable, ranging from 626 Kb to 2.57 Mb (supplementary Table I). The size of the SRO was 188 Kb and encompassed four genes: HNRNPU, FAM36A, NCRNA00201, and EFCAB2. The identification of a normal individual with a partial deletion of the EFCAB2 gene led us to consider as unlikely causative this gene as a cause of ID, thus refining the SRO to three genes: HNRNPU, FAM36A, and NCRNA00201 (Fig. 2). In a previous study, Matsuzaki et al. [2009] identified a deletion involving HNRNPU in three HAPMAP individuals of Yoruba Nigerian origin. In contrast, we obtained normal results with our targeted 60K array, excluding a deletion in the 1q44 region in these individuals and, thus, demonstrating that the deletions identified by Matsuzaki et al. [2009] were false positive results. Therefore, no deletion involving one of these three genes located in this 1q44 region has been observed within individuals of the general population (www.tcag.org).

mRNA expression studies

We showed that HNRNPU, FAM36A and NCRNA00201 were expressed in 6 different tissues (adult brain, heart, kidney, liver, cerebellum tissues and fetal brain tissue), with the strongest expression in the cerebellum (Fig. 3). The highest level of transcripts was obtained for NCRNA00201 in the cerebellum. As the strongest expression of these three genes were detected in cerebellum, we analyzed the expression of two control genes: SULF1 and SLC05A1. We obtained low expression levels in the cerebellum compared to other tissues for these genes, thus excluding a potential bias in our sample of RNA extracted from the cerebellum (data not shown).
Mutation screening

Direct sequencing of *HNRNPU* and *FAM36A* did not reveal any deleterious point mutations in the remaining allele of the patients with a 1q44 deletion, rendering unlikely a recessive mode of inheritance. Neither did we detect any deleterious mutations in these two genes in our series of 191 patients with unexplained ID. Two identified exonic variants in *8HNRNPU* (exon 6 c.1215G>A, synonymous; exon 14 c.2437C>G, p.Gln813Glu) and one in *9FAM36A* (exon 4 c.340G>A, p.Gly114Ser) were predicted to be benign using the PolyPhen software.

DISCUSSION

In this study, all eleven patients carrying a 1q44 microdeletion presented with moderate to severe ID, seizures and non-specific craniofacial anomalies, corresponding to a non-recognizable phenotype with ID. The aCGH data allowed us to fine map a SRO for moderate to severe ID and seizures. However, since other reports described patients with deletions in the 1q43q44 bands that did not include the SRO defined in the present study, there may be a number of additional genes that when haploinsufficient can cause ID in these patients.

Two recent studies, a first one performed on 22 patients [Ballif et al., 2011] and a second one based on 7 patients [Nagamani et al., 2012] sharing 1q43q44 microdeletion, clarified the phenotype/genotype correlation and proposed three distinct SRO. The first SRO encompassing *ZNF238* was associated with corpus callosum abnormalities, the second SRO including *AKT3* caused microcephaly in most patients while the third SRO containing the
three genes FAM36A, HNRNPU and NCRNA00201 was associated with seizures. In our study, 2/11 patients (#1 and #2) carried a deletion of both ZNF238 and AKT3. Both patients presented with microcephaly which was consistent with a role of AKT3 in microcephaly. Only patient 2 presented with a corpus callosum agenesis. Incomplete penetrance associated with deletion of ZNF238 could explain the lack of corpus callosum abnormality in patient 1. Finally, the third previously published SRO associated with seizures in Ballif et al. [2011] overlapped with our 188 Kb SRO associated with ID, seizures and craniofacial anomalies.

FAM36A encodes a hypothetical protein and, to date, its biological role is still unknown. HNRNPU is a protein-coding gene comprising 14 exons, which are highly conserved during evolution. The HNRNPU protein is able to bind RNAs and mediates different aspects of their metabolism and transport [Dreyfuss et al., 2002; Krecic and Swanson, 1999]. Mice with a homozygous hypomorphic mutation in HNRNPU are severely retarded in both growth and development indicating that this gene is essential for embryonic development [Roshon et al., 2005]. Although ubiquitously expressed, we detected the highest expression level for HNRNPU in human cerebellum, a tissue which plays an essential role in cognition. Interestingly, HNRNPU is involved in later stages of differentiation of cerebellar neurons via the regulation of DNA topoisomerase IIβ activity [Kawano et al., 2010]. Thus, haploinsufficiency for HNRNPU may lead to ID in our patients, even in the absence of clinical cerebellar anomalies. CDH15 is such an example where a gene is strongly expressed in the cerebellum and mutations for which are associated with ID with no features of cerebellar dysfunction [Bhalla et al., 2008].

The third gene within the SRO of our study, NCRNA00201, encodes a long non-coding RNA (IncRNA). The majority of IncRNA has very high levels of expression in the central nervous system in a cell-type specific manner, of which some have already been
implicated in neurological and developmental disorders [for a review Qureshi et al., 2010]. It is assumed that they regulate gene expression notably via chromatin remodeling at their originate locus (in cis) and/or elsewhere in the genome (in trans). This property considerably increases the difficulty to identify their triggers and to understand their physiological roles.

By RT-qPCR, we detected the highest expression level for NCRNA00201 in human cerebellum, making it a good candidate. Moreover, since our three deleted genes showed relatively high expression in the cerebellum when compared to other tissues, we might hypothesize that an epistatic effect of at least two genes from this locus could be responsible for our patients’ phenotype. The lack of knowledge and the difficulty to interpret the variants identified in a non-coding gene explain why we did not sequence NCRNA00201 in our series of patients with ID. NCRNA00201 still remains a good candidate to explain ID but functional analyses are needed to clarify the implication of this gene in the phenotype.

Taken together, our aCGH, expression and sequencing data highlight a critical region containing three good candidate genes for non-syndromic ID and seizures. These results will be important for clinicians in genetic counseling.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

4 REFERENCES


seizures and variable corpus callosum thickness sharing a 0.440 Mb deletion in region 1q44 containing the HNRPU gene. Eur J Med Genet 53:179-85.


2 LEGENDS TO FIGURES

4 Figure 1. Facial phenotypes of six patients with interstitial 1q44 deletion showing non-specific craniofacial anomalies.

7 Figure 2. A. Map of the deletions in chromosomal band 1q44 identified by aCGH. Black horizontal bars indicate the deletions in the 11 patients with ID and seizures. Grey horizontal bar (CNP) indicates the deletion that we have identified in a healthy individual. The RefSeq genes located in the genomic region are indicated. The vertical region shaded in red indicates the smallest region of overlap (SRO) implicated in ID and seizures from our study; in yellow, the SRO implicated in microcephaly; in blue, the SRO implicated in corpus callosum abnormalities. B. Detailed map of the proposed critical region for ID and seizures, which contains three candidate genes: HNRNPU, FAM36A and NCRNA00201. The EFCAB2 gene was considered as unlikely causative since we have identified it in a healthy individual. Horizontal red bars indicate the deletions reported in the Database of Genomic Variants (www.tcag.org). Three variants involving at least one of the three candidate genes (HNRNPU, FAM36A and NCRNA00201) are reported in the Database of Genomic Variants have been identified in three HAPMAP individuals of Yoruba Nigerian origin [Matsuzaki et al., 2009]. However, we obtained normal results with our targeted 60K array, excluding a deletion in the 1q44 region in these individuals and, thus, demonstrating that the deletions identified by Matsuzaki et al. [2009] were false positive results.
Figure 3. Expression patterns of HNRNPU, FAM36A and NCRNA00201 in a panel of human tissues.

cDNA were obtained using the MMLV reverse transcriptase (Invitrogen) with random primers from 1 µg of human total RNA of five adult and four fetal tissues. Real-time PCR was performed in triplicates using Takara SYBR premix on Light Cycler 480 (Roche diagnostics). The ∆∆Ct method was used to assess expression level of three target genes - HNRNPU, FAM36A and NCRNA00201 – relative to the expression level of the β-actin (ACTB) and cyclophilin E (PPIE) housekeeping genes. For a given target gene, the ∆Ct of each tissue was compared to the median of the ∆Ct of the 6 tissues analyzed.
TABLE I. Clinical features and array CGH data of the 11 patients with 1q44 microdeletion
<table>
<thead>
<tr>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
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<th>Patient 6</th>
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<td>6/12</td>
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<td>2 9/12</td>
<td>13 9/12</td>
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<td>7 1/12</td>
<td>4 10/12</td>
<td>9 10/12</td>
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<tr>
<td>Coordinates of the deletions (in Mb using hg19 genome build)</td>
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<td>244.3-245.1</td>
<td>244.4-245.3</td>
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<td>244.8-245.5</td>
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### Supplementary TABLE I. Boundaries and sizes of the deletions identified by array CGH

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