Overlapping cortical malformations and mutations in TUBB2B and TUBA1A

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Polymicrogyria and lissencephaly are causally heterogeneous disorders of cortical brain development, with distinct neuropathological and neuroimaging patterns. They can be associated with additional structural cerebral anomalies, and recurrent phenotypic patterns have led to identification of recognizable syndromes. The lissencephalies are usually single-gene disorders affecting neuronal migration during cerebral cortical development. Polymicrogyria has been associated with genetic and environmental causes and is considered a malformation secondary to abnormal post-migrational development. However, the aetiology in many individuals with these cortical malformations is still unknown. During the past few years, mutations in a number of neuron-specific \( \alpha \) - and \( \beta \)-tubulin genes have been identified in both lissencephaly and polymicrogyria, usually associated with additional cerebral anomalies including callosal hypoplasia or agenesis, abnormal basal ganglia and cerebellar hypoplasia. The tubulin proteins form heterodimers that incorporate into microtubules, cytoskeletal structures essential for cell motility and function. In this study, we sequenced the TUBB2B and TUBA1A coding regions in 47 patients with a diagnosis of polymicrogyria and five with an atypical lissencephaly on neuroimaging. We identified four \( \beta \)-tubulin and two \( \alpha \)-tubulin mutations in patients with a spectrum of cortical and extra-cortical anomalies. Dysmorphic basal ganglia with an abnormal internal capsule were the most consistent feature. One of the patients with a TUBB2B mutation had a lissencephalic phenotype, similar to that previously associated with a TUBA1A mutation. The remainder had a polymicrogyria-like cortical dysplasia, but the grey matter malformation was not typical of that seen in ‘classical’ polymicrogyria. We propose that the cortical malformations associated with these genes represent a recognizable tubulinopathy-associated spectrum that ranges from lissencephalic to polymicrogyric.
cortical dysplasias, suggesting shared pathogenic mechanisms in terms of microtubular function and interaction with microtubule-associated proteins.

Keywords: lissencephaly; polymicrogyria; corpus callosum; tubulinopathy; neuronal migration

Introduction

Families of tubulin genes encode α- and β-tubulin isotypes, sharing extremely high levels of sequence homology and subject to specific spatial and temporal expression patterns (Villasante et al., 1986; Wang et al., 1986). They encode α- and β-tubulins, ubiquitous proteins that dimerize to form αβ tubulin heterodimer complexes. These dimers incorporate into microtubule polymers, polarized cytoskeletal structures with the capability to dynamically depolymerize. This dynamic instability enables microtubules to perform various mechanical tasks, including maintenance of cell morphology, axon formation, mitosis and intracellular trafficking (Mitchison and Kirschner, 1984; Desai and Mitchison, 1997; Feng and Walsh, 2001; Guzik and Goldstein, 2004).

The microtubule network has highly specialized roles within neurons, especially during cerebral cortex development. Neurons require a bipolar morphology to migrate from proliferative zones in the developing forebrain to specified locations within the cortical plate (Marin et al., 2010). Polarized bundles of microtubule polymers generate neuronal processes that extend into the direction of migration, while a cage-like network of dynamic microtubules translocates the nucleus and associated organelles forward into this leading neurite (Ayala et al., 2007). Mutations in neuronally expressed tubulin genes (e.g. TUBA1A, TUBB2B, TUBB3 and TUBA8) have been identified in patients suffering from a range of cerebrocortical malformations. This supports that, despite their high sequence homology, they have subtle but distinct roles in microtubular dynamics and function (Tischfield and Engle, 2010).

Polymicrogyria is the most common malformation of cortical development. It is characterized by an excessive number of small and fused gyri, and associated with a thin and irregular cortex. On neuroimaging, the cortex appears thickened with an irregular cortical surface and grey-white matter junction (Leventer et al., 2010). Individuals with this disorder usually have psychomotor delay and a cognitive impairment, and ~80% will develop epilepsy (Leventer et al., 2010). The pathogenesis is heterogeneous and includes intrauterine hypoxia of the developing brain, congenital infection, chromosome anomalies and single-gene disorders. The latter include metabolic and known malformation syndromes, such as pyruvate dehydrogenase deficiency syndrome and Goldberg–Sphingten syndrome (Brooks et al., 2005; Judkins et al., 2011). The most common associated cytogenetic anomaly found with polymicrogyria is a chromosome 22q11 deletion (Robin et al., 2006). However, many disorders where polymicrogyria is the primary anomaly remain unexplained, although mutations in a number of genes, including SRPX2, TBR2, PAX6 and WDR62, have been identified in small subgroups of patients (Guerrini et al., 2008; Leventer et al., 2010; Murdock et al., 2011).

Based on their findings in a recent neuropathological study, the authors suggested polymicrogyria should be considered a post-migrational malformation of cortical development (Judkins et al., 2011; Barkovich et al., 2012).

The lissencephalies are considered neuronal migration disorders. Classical lissencephaly, the most common form, is predominantly caused by mutations in the LIS1 (also known as PAFAH1B1) and DCX genes. Lissencephaly with cerebellar hypoplasia has been associated with RELN and VLDLR mutations in a small subset of patients. In males with X-linked lissencephaly and ambiguous genitalia, variants in the ARX gene were identified (Kato et al. 2004; Guerrini et al., 2008). Keays et al. (2007) published the first association between mutations in an α-tubulin gene, TUBA1A, and lissencephaly. A comprehensive study of the phenotypes seen with TUBA1A mutations indicated that they are most commonly found in lissencephaly with cerebellar hypoplasia, but also in some patients with classical lissencephaly (Kumar et al., 2010). Recently, an inherited TUBA1A mutation was reported in two sisters with perisylvian focal polymicrogyria, and somatic mosaicism confirmed in their unaffected mother (Jansen et al., 2011).

Jaglin et al. (2009) described mutations in the β-tubulin gene, TUBB2B, in four patients with asymmetrical polymicrogyria recognized on post-natal neuroimaging in one foetus with a complex cortical malformation (Jaglin et al., 2009). Their review of brain pathology in the foetus showed some features consistent with unlayered polymicrogyria, but also showed radial columnar heterotopia and neuronal overmigration through the pial basement membrane, features that are not typical of any recognized form of polymicrogyria. Based on these (and other) observations, we prefer to designate this as atypical polymicrogyria or a polymicrogyria-like cortical malformation. Additional cerebral anomalies included abnormal basal ganglia and hypoplasia of corpus callosum, cerebellum and pons, features also noted in patients with TUBA1A mutations. Recently, a further four patients with TUBB2B mutations have been published: two with polymicrogyria and another with pachygyria (Guerrini et al., 2012) and one with polymicrogyria and a schizencephalic cleft (Romaniello et al., 2012).

Tubulin gene involvement also includes homozygous deletions detected in TUBB8 in four children from two consanguineous families with extensive polymicrogyria, callosal anomalies and optic nerve hypoplasia (Abdollahi et al., 2009). Mutations in TUBB3 were initially reported in congenital fibrosis of the extraocular muscles type 3 (Tischfield et al., 2010). Subsequently, de novo and familial TUBB3 mutations were also identified in children with cortical dysgenesis, but no congenital fibrosis of the extraocular muscles (Poirier et al., 2010).

In this study, we present our findings in 47 patients with a diagnosis of polymicrogyria on neuroimaging and five patients with a
lissencephalic phenotype, with or without associated cerebral malformations, who were screened for mutations in the TUBB2B and TUBA1A genes. We discovered four novel TUBB2B and two TUBA1A variations in six patients with a spectrum of overlapping cortical anomalies, and present in silico homology modelling to gain insights into the protein effects of mutations.

Materials and methods

Clinical ascertainment

We sequenced TUBB2B and TUBA1A in 47 subjects with polymicrogyria or polymicrogyria-like cortical malformations, and five with unexplained lissencephaly (negative for mutations in LIS1 and DCX).

While the cohort included all common subtypes of polymicrogyria, we weighted selection towards patients who also had associated callosal and cerebellar (n = 17) or only cerebellar (n = 3) hypoplasia, or a frontal-predominant gradient of the cortical malformation (n = 9). Additional clinical details were obtained for the six patients in whom TUBB2B or TUBA1A mutations were found. Their neuroimaging findings were reviewed and compared with the previously published images in association with mutations in any of four tubulin genes: TUBA1A, TUBB5, TUBB2B and TUBB3 (Keays et al., 2007; Poirier et al., 2007, 2010; Bahl-Buisson et al., 2008; Morris-Rosendahl et al., 2008; Abdollahi et al., 2009; Jaglin et al., 2009; Kumar et al., 2010; Tischfield et al., 2010; Jansen et al., 2011; Guerrini et al., 2012; Romaniello, 2012). TUBB2B was also sequenced in a cohort of 110 TUBA1A mutation-negative patients with lissencephaly, who were part of a previous study of the TUBA1A gene (Kumar et al., 2010). The research was approved by the Research Ethics Committee for Wales and Institutional Review Boards at the University of Chicago and Seattle Children’s Hospital.

DNA mutation analysis

DNA was extracted from peripheral blood using standard methods. DNA samples were screened for mutations in TUBB2B and TUBA1A genes (NCBI Accession: NC_000006.11 and NC_000012.11) using PCR (QIAGEN) and direct Sanger sequencing. PCR primers were designed using Primer3 (http://frdo.wi.mit.edu/primer3/) to amplify protein-coding regions and splice sites. All primers were located within intronic regions and avoiding single-nucleotide polymorphisms based on National Centre for Biotechnology Information polymorphism database (http://www.ncbi.nlm.nih.gov/snp). Primer sequences and PCR conditions are available on request to the corresponding author.

PCR products were purified using QIAquick® purification kits (QIAGEN) and sequenced using BigDye® terminators and an ABI3130 automated sequencer (PE Applied Biosystems). The presence of each variant detected was assessed in 100 normal control (200 chromosomes) samples using LightScanner® mutation analysis technology and checked against two online polymorphism databases, dbSNP (above) and the 1000 Genomes catalogue (www.1000genomes.org). We searched for all sequence variants in the Exome Variant Server, NHLBI GO Exome Sequencing Project (http://evs.gs.washington.edu/EVS/) (October, 2012), which contains whole exome sequencing data from 6503 individuals. The depth of coverage across these two genes ranged between 3269 and 13,005 reads for the synonymous variants shown in the database. SIFT (http://sift.jcvi.org/www/SIFT_seq_submit2.html) and Align GVGD (http://agvgd.iarc.fr/agvgd_input.php/) web-based programs were used to predict whether each variant would significantly affect protein function, based on multiple protein sequence alignments and biophysical characteristics of the substituted amino acids.

Homology modelling

Structural modelling of wild-type and mutant TUBA1A and TUBB2B protein subunits was carried out using a previously described homology modelling pipeline (Mullins et al., 2010). The best homology attained for each model was based on 100 and 40.7% identity, respectively, with the crystal structure of an α-tubulin template (PDB: 1JFF; Lowe et al., 2001).

Patients with TUBB2B mutations

Patient 1 (p.R380S) was a 12-year-old female, born at term. Her birth weight was on the 50th centile and her occipitofrontal circumference on the 75th centile. She developed post-natal microcephaly. Onset of seizures was at the age of 6 months. She also had global developmental delay, significant intellectual disability, optic atrophy and a thoracic scoliosis. Her brain MRI scan at the age of 20 months showed a bilateral asymmetrical irregular polymicrogyria-like gyral pattern in the entire perisylvian region and elongated sylvian fissures, with extension to the posterior temporal and anterior parietal lobe on the left. There was also an unusual, bilateral, midline suture pattern with a superomedia polymicrogyria-like cortex. The white matter was decreased with mildly enlarged bodies of lateral ventricles. Additional features included absence of the corpus callosum, a large cavum septum pellucidum, hypoplastic pons and cerebellar vermis, and normal cerebellar hemispheres. The basal ganglia were abnormally orientated with absence of the internal capsule (Table 1; Fig. 1G–I).

Patient 2 (p.R380C) was a 25-year-old female born at term to non-consanguineous parents. Her birth weight was between the 50th and 75th centiles, length on the 91st centile and occipitofrontal circumference between the 2nd and 9th centiles. Her development was globally delayed; she never walked and had severe intellectual disability and no speech. Epilepsy was diagnosed at the age of 11 months. The seizures were initially focal and subsequently generalized. She developed a scoliosis at the age of 5 years. Puberty was normal. At the age of 22 years, her occipitofrontal circumference was well below 0.4th centile. She appeared short, but she could not be formally measured. She had deep-set eyes, straight eyebrows, a prominent lower face, short philtrum, full lips, low posterior hairline and small hands and feet. At the age of 16 years, she was found to have a right unilateral complex, cystic, cerebellar-pontine angle Schwannoma on neuroimaging; tissue from the tumour did not reveal NF2 mutations. Her brain MRI scan also showed a bilateral, perisylvian polymicrogyria-like cortical dysplasia extending to the middle temporal lobes. The corpus callosum was absent, there was a persistent cavum septum pellucidum and the periventricular white matter was markedly reduced. The brainstem was small and cerebellar vermis hypoplastic (Table 1; Fig. 1J–L).

Patient 3 (p.L207P) was a 12-year-old male, born at term. His birth weight was on the 25th centile, length on the 91st centile and occipitofrontal circumference between the 0.4th and 2nd centiles. Enlarged lateral cerebral ventricles had been noted during pregnancy. He developed seizures at the age of 4 months; he had occasional generalized tonic-clonic seizures and frequent myoclonic and tonic seizures before going to sleep. He had severe psychomotor delay, profound intellectual disability and poor eye contact. He developed...
### Table 1
Summary of findings in our patient cohort and published patients with polymicrogyria and TUBB2B and TUBA1A variants

<table>
<thead>
<tr>
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<tr>
<td>Mutation</td>
<td>R380S</td>
<td>R380C</td>
<td>L207P</td>
<td>G98R</td>
<td>T312M</td>
<td>L228P</td>
</tr>
<tr>
<td>Nucleotide</td>
<td>1138C</td>
<td>620T</td>
<td>292G</td>
<td>935C</td>
<td>629T</td>
<td>1249G</td>
</tr>
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<td>Inheritance</td>
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<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<tr>
<td>Age</td>
<td>12 years</td>
<td>25 years</td>
<td>12 years</td>
<td>10 years</td>
<td>2 years</td>
<td>2 years</td>
</tr>
<tr>
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<td>FM</td>
<td>M</td>
<td>M</td>
<td>MFM</td>
<td>M</td>
</tr>
<tr>
<td>Microcephaly</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Eyes</td>
<td>OA</td>
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<td>-</td>
<td>NA</td>
<td>?</td>
<td>?</td>
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<tr>
<td>Seizures</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Onset</td>
<td>6 months</td>
<td>11 months</td>
<td>4 months</td>
<td>NA</td>
<td>3 months</td>
<td>?</td>
</tr>
<tr>
<td>Scoliosis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Psychomotor delay</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cortex</td>
<td>DPMG</td>
<td>PMG</td>
<td>PMGL</td>
<td>ex-tending</td>
<td>into frontal and parietal lobes on the left</td>
<td>ex-tending into medial temporal lobes</td>
</tr>
<tr>
<td>Agyria and thick, irregular subcortical band of grey matter</td>
<td>DPMG</td>
<td>PMG; predom. in left frontal and parietal lobes</td>
<td>PMG; predom. frontal and temporal lobes</td>
<td>PMG; predom. left frontal, parietal and temporal lobes</td>
<td>DPMG; predom. in perisylvian regions</td>
<td></td>
</tr>
<tr>
<td>Posterior cerebral cortex</td>
<td>DPMG/SCZ</td>
<td>PMG</td>
<td>PMGL</td>
<td>Right unilateral focal PMGL</td>
<td>PSPMG</td>
<td>PSPMG</td>
</tr>
<tr>
<td>Bilateral asymmetry</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Corpus callosum</td>
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<td>ACC</td>
<td>ACC</td>
<td>ACC</td>
<td>CCHL</td>
<td>ACC</td>
</tr>
<tr>
<td>Enlarged LVENTs</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Basal ganglia</td>
<td>dysmorph</td>
<td>dysmorph</td>
<td>dysmorph</td>
<td>dysmorph</td>
<td>dysmorph</td>
<td>dysmorph</td>
</tr>
<tr>
<td>Cerebellum</td>
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<td>CVHL</td>
<td>CBHL</td>
<td>Mild CBHL</td>
<td>CVHL</td>
<td>CVHL</td>
</tr>
<tr>
<td>Brainstem</td>
<td>BSHL</td>
<td>BSHL</td>
<td>BSHL</td>
<td>BSHL</td>
<td>Mild BSHL</td>
<td>BSHL</td>
</tr>
</tbody>
</table>

This table does not include the foetus included in Jaglin et al., 2009.

+ = only one parent available for parental testing; ACC = absence of the corpus callosum; BSHL = brainstem hypoplasia; CBHL = cerebellar hypoplasia; CCHL = hypoplastic corpus callosum; CVHL = cerebellar vermis hypoplasia; d = deceased; dn = de novo; DPMG = diffuse polymicrogyria; DPMGL = diffuse polymicrogyria-like; LVENTs = lateral ventricles; mat mos = maternal mosaicism; NA = not applicable; OA = optic atrophy; PCSP = persistent cavum septum pellucidum; PMG = polymicrogyria; PMGL = polymicrogyria-like; PSPMG = perisylvian polymicrogyria; PSPMGL = perisylvian polymicrogyria-like; SCZ = schizencephaly; SP = septum pellucidum.

Table 2 from Tubulinopathies and cortical malformations Brain 2013: 136; 536–548 | 539
by guest on November 7, 2016 | Downloaded from http://brain.oxfordjournals.org/ | guest on November 7, 2016
progressive microcephaly, with an occipitofrontal circumference well below the 0.4th centile at 4 months and at 7 years of age. He had a spastic tetraparesis and a positional scoliosis, which became apparent at the age of 1.5 years. He had a tilted head position, which resulted in facial and skull asymmetry. He also had a sloping forehead, dysplastic helices and anteverted nares. His brain MRI at age 4 months had the appearance of nearly complete agyria, with a thin cortex and a thick undulating irregular band of subcortical ectopic grey matter. There was absence of the corpus callosum, absent internal capsules, and there was unusual orientation of the basal ganglia and thalami. The brainstem was thin; there was cerebellar hypoplasia, with a less hypoplastic vermis (Table 1; Fig. 1A–C).

Patient 4 (p.G98R) was a female, born at term; her birth weight was on the 25th centile and her length on the 75th centile. An accurate neonatal head circumference measurement was not available. At the age of 3 months, she developed seizures. Delayed development and a small head size were noted. Optic disc pallor was identified and a subsequent diagnosis of bilateral optic atrophy was made. By the age of 5 years, she had developed a scoliosis. Because of feeding problems, a gastrostomy was inserted. She had severe psychomotor delay and had also developed an obstructive sleep apnoea. Her occipitofrontal circumference at 2 years and 2 months was well below the 0.4th centile, her height on the 91st centile and weight between the 9th and 25th centiles. Her cerebral MRI scan at 2 years and 2 months showed a diffuse cortical dysplasia with undersulcation and a variably thick polymicrogyria-like cortex, more severe in the posterior frontal lobes, perisylvian regions and temporal lobes. The white matter volume was markedly decreased, with moderately enlarged ventricles, especially the frontal horns. There was a diffusely increased white matter signal. The corpus callosum was absent. The hippocampi were small and dysplastic, and the brainstem moderately thin. There was mild cerebellar vermis and cerebellar hemisphere hypoplasia. The basal ganglia were abnormally orientated with absence of the internal capsule (Table 1; Fig. 1D–F).

Patients with TUBA1A mutations

Patient 5 (p.L70S) was a female born at term with an occipitofrontal circumference between the 2nd and 9th centiles. An eye examination showed bilateral optic nerve hypoplasia. She had a severe neurodevelopmental disorder. She died in infancy. A foetal MRI brain scan was reported as showing lissencephaly, absence of the corpus callosum, large lateral ventricles and a small cerebellum. Her post-natal MRI scan at the age of 8 days showed a very immature looking brain with a diffusely reduced gyral pattern, a thin and disorganized polymicrogyria-like cortex, more marked in the posterior perisylvian regions, and extended sylvian fissures. The lateral ventricles were markedly enlarged, with significant enlargement of the temporal horn. The cerebral white matter was markedly reduced. The corpus callosum was absent. The hippocampi were small and dysplastic, and the brainstem moderately thin. There was mild cerebellar vermis and cerebellar hemisphere hypoplasia. The basal ganglia were abnormally orientated with absence of the internal capsule (Table 1; Fig. 1M and N).

Figure 1 Brain MRI images. (A–L) Patients with TUBB2B mutations [(A–C) Patient P3, (D–F) Patient P4, (G–I) Patient P1, (J–L) Patient P2] and (M–Q) TUBA1A mutations [(M and N) Patient P5, (O–Q) Patient P6] in order of severity of neuroradiological findings and lissencephalic (A–C; Patient P3) versus polymicrogyric phenotype (D–Q; Patients P4, P1, P2, P5 and P6); T1- and T2-weighted images. (A, D, G, J and O) Sagittal images (not available in Patient P5) showing absence of the corpus callosum (A, D, G and J) and hypoplasia of the corpus callosum (O); mild to severe hypoplasia of the brainstem and cerebellar vermis, and a large tectum, especially in Patient P1 (A) and Patient P4 (D) (white arrowheads). (B) Axial image showing generalized agyria and bilateral irregular bands of subcortical grey matter (white arrows). (E, H, K, M and P) Axial images showing a diffuse (E and M) or focal (H, K, P and Q) polymicrogyria-like cortical dysplasia (white arrows). (C, F, I, L and Q) Axial images showing dysmorphic basal ganglia associated with an abnormal internal capsule. (N) Coronal image showing the cortical dysplasia in Patient P5.
Patient 6 (p.A333V) was a 7-year-old male, who was one of non-identical twins. There were no concerns about his twin brother. He was born at 33 weeks gestation. His birth weight was between the 25th and 50th centiles, and occipitofrontal circumference was between the 75th and 91st centiles. He had a longstanding history of locomotor problems; he sat at the age of 2.5 years, walked at the age of 5 years and his coordination was poor. Speech development was also delayed, but by the age of 7 years he was talking in simple sentences. He had problems with chewing. A convergent squint was surgically corrected. He had moderate learning difficulties and a poor attention span. He had developed myoclonic non-epileptiform jerks at the age of 5 years. When assessed at the age of 7 years, his occipitofrontal circumference was on the 50th centile and he was non-dysmorphic. His right leg was congenitally shorter than his left leg, and he had a postural mild lumbar scoliosis. He also had joint laxity and a generally decreased tone. As he had been noted to have ventriculomegaly antenatally, a brain MRI scan was arranged at the age of 3 weeks and repeated at the age of 5 years. It showed a few, highly focal areas of an irregular polymicrogyria-like cortex. The corpus callosum was thin, and the anterior limbs of the internal capsule were absent, as was the septum pellucidum. The fourth ventricle was enlarged in association with pontocerebellar hypoplasia (Table 1; Fig. 1O–Q).

Results

Genetic screening

Forty-seven patients with polymicrogyric phenotype and five patients with a lissencephalic cortical dysplasia were screened for TUBB2B and TUBA1A sequence variations. Four novel heterozygous mutations were identified in exon 4 of TUBB2B: c.292 G > A (p.G98R); c.620 T > C (p.L207P); c.1138 C > A (p.R380C) and c.1138 C > T (p.R380S). In addition, two novel mutations were detected in TUBA1A: c.209 T > C (p.L70S) and c.1001 C > T (p.A333V) (Fig. 2A; Table 1). Each variant identified translates into a missense mutation affecting highly conserved residues of the β- and α-tubulin proteins (Fig. 2C). All mutations were confirmed as de novo following exclusion from parental samples and absence confirmed in 100 control samples as well as online polymorphism databases (www.ncbi.nlm.nih.gov/projects/SNP/ and www.1000genomes.org). Furthermore, none of the six sequence changes in TUBA1A or TUBB2B (or any other non-synonymous sequence changes) were found in the NHLBI Exome Variant Server maintained at the University of Washington (http://evs.gs.washington.edu/EVS/). They were all predicted to be pathogenic variations by SIFT and Align GVGD mutation prediction software (http://sift.jcvi.org/www/SIFT_seq_submit2.html and http://agvgd.iarc.fr/agvgd_input.php/). To further elucidate the phenotypic spectrum of TUBB2B mutations, we also sequenced the gene in 110 TUBA1A mutation negative patients with lissencephaly. No non-synonymous sequence variants in TUBB2B were detected in this cohort.

TUBB2B phenotypes

Four novel TUBB2B missense mutations (7.7%) were identified in our cohort of 52 patients, representing a relatively wide spectrum of polymicrogyria and five with a lissencephalic phenotype. All four patients had severe psychomotor delay and intellectual disability, epilepsy with onset of seizures before the age of 12 months and post-natal microcephaly in three of four patients (data not available in Patient P4). Two patients also had optic atrophy (Patients P1 and P4). Scoliosis with onset in early childhood was present in all four patients, but not associated with structural spinal anomalies and likely to be postural (Table 1). Two of the patients (Patients P2 and P3) also had some non-overlapping dysmorphic facial features.

Neuroimaging showed a predominantly perisylvian polymicrogyria-like cortical dysplasia in Patient P1 (Fig. 1G–I) and Patient P2 (Fig. 1J–L). The corpus callosum was absent in both, and the brainstem and cerebellar vermis were small. The basal ganglia had an unusual orientation. Their similar neuroimaging findings are associated with a mutation in the same amino acid residue. Patient P4 (Fig. 1D–F) had a diffuse polymicrogyria-like cortical dysplasia, an absent corpus callosum and an abnormal orientation of the basal ganglia. Patient P3 (Fig. 1A–C) had a lissencephalic phenotype with an agyric cortex and a bilateral, subcortical, irregular band of grey matter. The basal ganglia were dysmorphic with absence of the internal capsule. The corpus callosum was absent and the hypoplasia of the cerebellum was more marked than in the other patients.

We also identified a maternally inherited TUBB2B variant, c.1063 G > A (p.D355N), in a female neonate born at 35 weeks gestation, who died on Day 1 of life secondary to pulmonary hypoplasia. There was no evidence of maternal mosaicism on Sanger sequencing. Post-mortem examination revealed generalized arthrogryposis and micrognathia with a posterior cleft palate. Neuropathological examination showed bilateral polymicrogyria, severe cerebellar and brainstem hypoplasia and spinal cord atrophy. There were also signs of white matter damage and periventricular calcification, which could suggest an in utero insult. Arthrogryposis has been observed in patients with sporadic polymicrogyria, but not in patients with TUBB2B mutations (Jaglin et al., 2009; Guerrini et al., 2012; Romaniello et al., 2012). Considering the post-mortem findings and that the TUBB2B variant in this patient was maternally inherited, it seems unlikely to be pathogenic. Although the base pair change was absent in 100 controls, on NCBI dbSNP, 1000 Genome Project Database and the NHLBI Exome Variant Server, online mutation prediction software (SIFT and Align GVGD) suggests that this variant is relatively benign and would not cause significant effects on protein function. Therefore, we currently consider it a rare polymorphism in the population.

TUBA1A phenotypes

We identified TUBA1A variants in 2 of 52 individuals (3.8%). One of the patients (Patient P5) had a severe neurodevelopmental disorder and optic nerve hypoplasia. On review, the features on her brain MRI scan were most consistent with a diffuse polymicrogyria-like cortical dysplasia. The other patient (Patient P6) had an unusually mild clinical phenotype associated with a predominantly normal cortex and only highly localized areas of irregular cortex. The main features, reminiscent of a tubulinopathy, were the abnormal basal ganglia and cerebellar hypoplasia.
TUBB2B and TUBA1A negative patients

Of the remaining 46 TUBB2B and TUBA1A negative patients, 42 had polymicrogyria and four had a lissencephalic phenotype. The cortical anomalies ranged from unilateral and bilateral symmetric or asymmetric polymicrogyria. Hypoplasia or absence of the corpus callosum and hypoplasia of the cerebellum was seen in 11 and cerebellar hypoplasia alone in three individuals.

Homology modelling

We looked at the effect of the TUBB2B and TUBA1A mutations identified in this study and positioning of previously reported TUBB2B variants (Jaglin et al., 2009; Guerrini et al., 2012; Romaniello et al., 2012) and the TUBA1A mutation associated with polymicrogyria (Jansen et al., 2011). The positions of each amino acid substitution are indicated in relation to a predicted 3D
model of an α/β-tubulin heterodimer within a microtubule polymer structure (Fig. 3).

The TUBB2B R380 residue is located on the 11th α-helix of the protein subunit. Owing to their external position when polymerized within microtubules, α-helices 11 and 12 of both α- and β-tubulin protein subunits are sites of interaction with microtubule-associated proteins. TUBB2B R380 is predicted to bridge with the T253 residue of the β-tubulin of an adjacent heterodimer (Figs 3 and 4). This glycine is one of three residues forming a salt bridge between dimers, and the introduction of an arginine residue at position 98 the interaction between these tubulin proteins due to steric conflicts. This would suggest that the polymerization dynamics of microtubules might be impaired in vivo.

In comparison with both TUBB2B R380 and G98, the L207 residue is not located on the surface of the β-tubulin subunit (Fig. 3). Instead, it can be found towards the core of the globular protein. This may suggest an effect on microtubule dynamics secondary to conformational changes in tubulin subunits rather than an association with impaired protein–protein interactions.

In terms of TUBA1A, both the A333 and L70 residues are in relative proximity to the α/β-tubulin interfaces, as is the previously described I5 substitution (Jansen et al., 2011). The A333 is at the interdimer interface and interacts with I176 and V177 (Nogales et al., 1999). L70, however, is at the intradimer interface, buried within the α-tubulin GTP-binding fold (Nogales et al., 1998).

Discussion

The cortical malformations first associated with TUBA1A mutations were classified as lissencephaly and those with TUBB2B mutations as polymicrogyria (Keas et al., 2007; Jaglin et al., 2009). This distinction has been lost, however, as mutations of both TUBA1A and TUBB2B have now been associated with both lissencephaly and polymicrogyria (Jansen et al., 2011; Guerrini et al., 2012). To evaluate this connection, we reviewed available brain images of patients in our cohort and published patients with mutations of TUBA1A and TUBB2B, as well as the limited images available for children with mutations of TUBA8 and TUBB3 (Abdollahi et al., 2009; Poirier et al., 2010).

In children with cortical malformations resembling polymicrogyria, we found that the cortical surface and cortical–white matter boundary appear smooth in some areas and pebbled in others. The cortex is moderately thick (usually 7–10 mm), but...
the irregular intracortical microsulci typical of classic polymicrogyria are sparse or not seen. Further, histopathological review of the brain of a 27-week gestation foetus with a TUBB2B mutation was classified as unlayered polymicrogyria (Jaglin et al., 2009). At least two of the features shown, radial columnar heterotopia and neuronal overmigration through the pial basement membrane, are not typical of any recognized form of polymicrogyria. These features also overlap with the cobblestone cortical malformations seen in the dystroglycanopathies, muscle–eye–brain disease and Walker–Warburg syndrome (Manzini et al., 2008). In utero
knockdown of rat Tubb2b expression using RNA interference also results in disruption of neuronal migration, indicating that the pathomechanism of tubulin-associated polymicrogyria in humans is distinct from classic post-migrational polymicrogyria (Jaglin et al., 2009; Judkins et al., 2011). These findings are consistent with the neuroimaging appearances, which are not typical of classical polymicrogyria, and are best described as a polymicrogyria-like malformation.

The most common polymicrogyria-like cortical malformation found with TUBB2B mutations is characterized by a somewhat thicker cortex than seen in classic polymicrogyria, and resembles a subset of patients with TUBA1A mutations previously classified as ‘lissencephaly with cerebellar hypoplasia group 3’ (Kumar et al., 2010), as well as the cortical malformations shown in the few available patients with TUBA8 or TUBB3 mutations. Further overlap between the cortical malformations associated with TUBB2B and TUBA1A mutations is demonstrated by the neuroimaging findings in one of our patients (Patient P3) with TUBB2B mutation, which is consistent with the phenotype reported in patients with TUBA1A mutations classified as ‘severe lissencephaly with cerebellar hypoplasia group 4’ (Kumar et al., 2010).

Based on our review, we propose that the imaging and pathological features define a novel ‘polymicrogyria-like’ cortical malformation that merges with lissencephaly (pachygyria and agyria) as it becomes more severe, and that malformations along this spectrum are seen with mutations of all four tubulin genes reported to date, with TUBA1A mutations clustering at the severe end. The complete spectrum may be divided into five phenotypic groups in ascending severity, all associated with dysmorphic basal ganglia, hypoplasia or agenesis of the corpus callosum, and variable brainstem and cerebellar hypoplasia. The groups include Group 1, mildly simplified gyral pattern with areas of subtle irregular cortex; Group 2, perisylvian predominant/focal polymicrogyria-like cortical malformation; Group 3, diffuse polymicrogyria-like cortical malformation (cortex usually 7–10 mm); Group 4, lissencephaly grade 4 (pachygyria with cortex usually 10–15 mm) that may be diffuse but most severe in the perisylvian region, or restricted to the parietal region, and may have a mildly irregular brain surface in some areas as well as mild asymmetry; and Group 5, severe lissencephaly (agyria) resembling two-layered lissencephaly.

Group 1 includes Patient LR09-250 shown in the supplementary data in Kumar et al., (2010) and our Patient P6, both with TUBA1A mutations. Group 2 includes Patients P1 and P2 with TUBB2B mutations as well as, for example, the two sisters reported by Jansen et al. (2011), with a TUBA1A mutation. Group 3 includes Patients P4 and P5 with a diffuse polymicrogyria-like cortical dysplasia. Group 4 includes patients classed as Group 3 by Kumar et al. (2010), who have atypical pachygyria, frequent asymmetry and subtle irregularity of the cortex and, for example, the patient with pachygyria reported by Guerrini et al. (2012) with a TUBB2B mutation. Group 5 includes Patient P3 with a TUBB2B mutation, and the patients with TUBA1A mutations classed as Group 4 by Kumar et al. (2010).

A specific subset of patients with TUBA1A mutations were excluded from the above classification. They all have classical lissencephaly on neuroimaging, largely indistinguishable from the phenotype associated with LIS1 mutations, and an alteration in the same codon, R402 (Keays et al., 2007; Poirier et al., 2007; Morris-Rosendahl et al., 2008; Bahi-Buisson et al., 2009; Kumar et al., 2010). Therefore, this appears to be a classical lissencephaly associated with one specific TUBA1A mutation. Further evidence for this is provided from the neuropathological examinations in four foetuses with different TUBA1A mutations (Fallet-Bianco et al., 2008). The findings in one case with a R402C variant differed from the other three and were reminiscent of features observed in classical lissencephaly; the internal capsule looked normal. Macrocephaly was reported in this 35-week foetus, and congenital macrocephaly appears more common in patients with a mutation in codon R402 than in those with a LIS1 mutation (Daniela Pilz; personal observation).

Most similar to the neuroimaging findings in patients with tubulin gene mutations is a patient with compound heterozygosity of PAX6 mutations (Solomon et al., 2009). In addition to bilateral microphthalmia, he also had a complex cerebral anomaly with...
absence of the corpus callosum and hypoplastic brainstem and cerebellar vermis. The brain MRI images presented in the article also suggest a cortical dysplasia. PAX6 is part of a transcription factor sequence, Pax6→Tbr2→NeuroD→Tbr1, shown to be involved in the differentiation of radial glial cells to post-mitotic projection neurons (Englund et al., 2005; Hevner et al., 2006). Homozygous TBR2 silencing has been reported in a few individuals with a bilateral polymicrogyric cortex, absence of the corpus callosum and cerebellar hypoplasia (Baala et al., 2007). The cortical-cerebral findings in this pathway overlap with those associated with tubulin gene mutations.

Consistent with previously published TUBB2B mutations, all genomic variations in our study were heterozygous, de novo and located within the fourth exon. They all translate into missense mutations affecting highly conserved residues. Psychomotor delay, intellectual disability and microcephaly were also reported in all previously published patients (Jaglin et al., 2009; Guerrini et al., 2012; Romaniello et al., 2012), as well as seizures in five of eight patients. The age-of-onset of epilepsy was only available in three of eight patients, and also below the age of 12 months (Table 1).

Common features in this study and in previously published patients appear to be mildly enlarged and often asymmetric lateral ventricles, severe hypoplasia or absence of the corpus callosum, dysmorphic basal ganglia with a poorly defined internal capsule, hypoplasia of the brainstem including mildly flat ventral pons, mildly enlarged tectum and moderate to severe cerebellar/cerebellar vermis hypoplasia (Jaglin et al., 2009; Guerrini et al., 2012, Romaniello et al., 2012). Dysmorphosis or unusual orientation of the basal ganglia was seen in most patients (Table 1). Therefore these features, in addition to a dysplastic cortex, are an indication of a TUBA1A or TUBB2B mutation-associated phenotype. The functional overlap of these two genes also suggests that they share a common pathophysiological pathway (Jaglin and Chelly, 2009).

TUBA1A mutations had not been previously associated with polymicrogyria until a recent report described two sisters with perisylvian polymicrogyria, dysgenesis of the internal capsule and hypoplasia of the corpus callosum, brainstem and cerebellum (Jansen et al., 2011). They had an ISL1 mutation in exon 2 of TUBA1A, which was inherited from their unaffected mother, who was a somatic mosaic carrier of the mutation. One of the siblings also had optic nerve hypoplasia. Our two patients with TUBA1A mutations were screened as part of our TUBB2B-negative polymicrogyria cohort. Patient P6 only had a few discrete areas of a polymicrogyria-like cortical dysplasia, and the main feature of a tubulopathopathy was the dysmorphic basal ganglia. To our knowledge, this is the mildest phenotype, clinically and radiologically, so far described in association with a TUBA1A mutation. We also previously reported a patient with absence of the corpus callosum and a mutation in this gene (Kumar et al., 2010). The latter suggests that a cortical malformation is not obligatory in patients with a TUBA1A mutation.

Three of our patients had optic nerve hypoplasia (Patients P1, P4 and P5), also reported in one of the siblings with a TUBA1A mutation and polymicrogyria (Jansen et al., 2011). This feature was a consistent finding in the patients with TUBA8 variations (Abdollahi et al., 2009) and also reported in a foetus with a TUBB3 mutation (Poirier et al., 2010). Optic atrophy would be consistent with impaired microtubule-dependant axon guidance and maintenance.

Apart from patients with mutations in the same codon in TUBB2B and TUBA1A genes having similar neuroimaging features, there is no clear evidence of a specific genotype-phenotype correlation. The corticocerebral anomalies in Patients P1 and P2 are similar, and both have amino acid substitutions in the R380 residue of the β-tubulin, pointing towards a common pathogenesis. This arginine is highly conserved through both α- and β-tubulins (Nogales et al., 1998), suggesting it is critical to protein function (Fig. 2C). Interestingly, mutations affecting the equivalent arginine residue in both TUBA1A (R390C; Kumar et al., 2010) and TUBB3 (R380C; Tischfield et al., 2010) have been described in patients with absence of the corpus callosum, and in the case of the TUBB3 mutation, the ocular motility disorder congenital fibrosis of the extraocular muscles type 3. Our modelling suggests that mutations in the TUBB2B R380 residue affect microtubule–DCX interactions (Figs 4 and 5). DCX is a structural microtubule-associated protein that significantly controls and stabilizes stochastic microtubule dynamics (Moores et al., 2006). It is expressed in neurons during stages of cortical development and is critical to correct neuronal migration. Mutations in the X-linked DCX gene are associated with lissencephaly and subcortical band heterotopia (Guerrini et al., 2008). It has not, however, been previously linked to a polymicrogyria-like phenotype.

We predict that TUBB2B G98R results in impaired docking of α-tubulin at the interdimer interface owing to steric conflicts between the large arginine side chain with that of β-tubulin. Despite remaining within range of the β-subunit, the E254 residue of α-tubulin moves out of interaction range with the β-tubulin bound GTP molecule. This glutamate residue normally catalyses the hydrolysis of this GTP (Nogales et al., 1999), involved in regulating dynamic instability of microtubules in vivo. Patient P3 with the L207P mutation had the most severe brain abnormalities with a TUBB2B mutation in this study. One could postulate that mutations directly affecting the structural integrity of tubulin proteins produce a more severe outcome (e.g. TUBB2B L207P, G98R) than those that appear to affect microtubule dynamics indirectly through impaired microtubule-associated protein interaction (e.g. R380C&S).

Non-synonymous sequence changes in TUBA1A and TUBB2B are likely to be deleterious based on the high conservation scores across the entire genes, and on the lack of any non-synonymous variants in 6503 individuals in the NHLBI Exome Variant Server. Similarly, no deletions of either tubulin gene have been described.

In conclusion, mutations in neurally expressed tubulin genes, TUBA1A and TUBB2B, are associated with a wide spectrum of overlapping cortical malformations, ranging from classical lissencephaly to a polymicrogyria-like dysplasia. Similar cortical malformations have been reported in patients with TUBA8 and TUBB3 mutations. Anomalies of the corpus callosum, basal ganglia, cerebellum and brainstem are the most consistent features seen in these cerebral tubulopathies. In view of our findings, we have proposed five groups delineating the cortical malformations and a subset with classical lissencephaly.
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