Polyhydramnios, megalencephaly and symptomatic epilepsy caused by a homozygous 7-kilobase deletion in \textit{LYK5}

Erik G. Puffenberger,\textsuperscript{1,*} Kevin A. Strauss,\textsuperscript{1,*} Keri E. Ramsey,\textsuperscript{2} David W. Craig,\textsuperscript{2} Dietrich A. Stephan,\textsuperscript{2} Donna L. Robinson,\textsuperscript{1} Christine L. Hendrickson,\textsuperscript{1} Steven Gottlieb,\textsuperscript{3} David A. Ramsay,\textsuperscript{4} Victoria M. Siu,\textsuperscript{5} Gregory G. Heuer,\textsuperscript{6} Peter B. Crino\textsuperscript{7} and D. Holmes Morton\textsuperscript{1}

\textsuperscript{1}Clinic for Special Children, Strasburg, PA, USA, \textsuperscript{2}Neurogenomics Division, Translational Genomics Research Institute, Phoenix, AZ, USA, \textsuperscript{3}Pediatric Neurology, Lancaster General Hospital, Lancaster, PA, USA, \textsuperscript{4}Department of Pathology, London Health Sciences Centre, London, Ontario, Canada, \textsuperscript{5}Medical Genetics Program, London Health Sciences Centre, London, Ontario, Canada, \textsuperscript{6}Department of Neurosurgery, University of Pennsylvania, Philadelphia, PA, USA and \textsuperscript{7}Department of Neurology, University of Pennsylvania, Philadelphia, PA, USA

*These authors contributed equally to this work.

Correspondence to: Kevin A. Strauss, MD, Clinic for Special Children, 535 Bunker Hill Road, Strasburg, PA 17579, USA  
E-mail: kstrauss@clinicforspecialchildren.org

We used single nucleotide polymorphism (SNP) microarrays to investigate the cause of a symptomatic epilepsy syndrome in a group of seven distantly related Old Order Mennonite children. Autozygosity mapping was inconclusive, but closer inspection of the data followed by formal SNP copy number analyses showed that all affected patients had homozygous deletions of a single SNP (rs721575) and their parents were hemizygous for this marker. The deleted SNP marked a larger deletion encompassing exons 9–13 of \textit{LYK5}, which encodes STE20-related adaptor protein, a pseudokinase necessary for proper localization and function of serine/threonine kinase 11 (a.k.a. LKB1). Homozygous \textit{LYK5} deletions were associated with polyhydramnios, preterm labour and distinctive craniofacial features. Affected children had large heads, infantile-onset intractable multifocal seizures and severe psychomotor retardation. We designated this condition PMSE syndrome (polyhydramnios, megalencephaly and symptomatic epilepsy). Thirty-eight percent (\textit{N} = 16) of affected children died during childhood (ages 7 months to 6 years) from medical complications of the disorder, which included status epilepticus, congestive heart failure due to atrial septal defect and hypernatremic dehydration due to diabetes insipidus. A single post-mortem neuropathological study revealed megalencephaly, ventriculomegaly, cytomegaly and extensive vacuolization and astrocytosis of white matter. There was abundant anti-phospho-ribosomal S6 labeling of large cells within the frontal cortex, basal ganglia, hippocampus and spinal cord, consistent with constitutive activation of the mammalian target of rapamycin (mTOR) signalling pathway in brain.

**Keywords:** symptomatic epilepsy; mammalian target of rapamycin; Mennonite; single nucleotide polymorphism; syndromic developmental delay; tuberous sclerosis complex

**Abbreviations:** GFAP = glial acidic fibrillary protein; LFB-CV = luxol fast blue-cresyl violet; mTOR = mammalian target of rapamycin; P-S6 = phospho-ribosomal S6 protein; SNP = single nucleotide polymorphism; STK11 = serine/threonine kinase 11 (a.k.a. LKB1); STRAD = STE20-related adaptor protein; TSC = tuberous sclerosis complex


**Introduction**

Developmental delay (Battaglia and Carey, 2003; Shevell et al., 2003) is the presenting problem for 35% of the approximately 125 Amish and Mennonite patients evaluated each year at the Clinic for Special Children. Population-based genetic knowledge allows us to reach an aetiological diagnosis for some of these children (Morton et al., 2003; Strauss et al., 2005, 2006), but many do not have a recognizable clinical syndrome and remain without a diagnosis after exhaustive biochemical and cytogenetic testing (Hunter, 2000; Battaglia and Carey, 2003). Although some consider brain MRI informative in...
such cases (Shevell et al., 2003; Srour et al., 2006), we find that abnormal imaging results rarely imply a specific diagnosis.

In the present work, we describe the focused application of DNA microarrays to investigate a small cluster of Mennonite patients with syndromic epilepsy and developmental delay. In 1998, we evaluated a Mennonite boy born with a large head and distinctive facial features. He began to have intractable multifocal seizures at 4 months of age, and his subsequent developmental progress was stagnant. A brain MRI showed ventriculomegaly, focal subependymal dysplasia and multiple areas of high diffusion within subcortical white matter (Fig. 2). There was no specific genetic diagnosis after 6 years of detailed investigations.

In autumn of 2004, the boy’s mother identified several other Mennonite children with similar physical features, early onset focal seizures and severe psychomotor retardation. We collected DNA from seven individuals with a common phenotype and, using a 10,000 single nucleotide polymorphism (SNP) mapping strategy (Puffenberger et al., 2004; Strauss et al., 2005, 2006), found a homozygous deletion of a single SNP in all affected individuals. The deleted SNP marked a large deletion of LYK5, which encodes the STE20-related adaptor protein (STRAD) important for normal in vitro localization and catalytic activity of serine/threonine kinase 11 (STK11, a.k.a. LBK1) (Baas et al., 2003; Boudeau et al., 2003). This is the first description of a human disease associated with a mutation in LYK5. The neurological phenotype implies a specific role for STRAD in human brain growth and development, and a more general role for the mTOR signalling pathway in certain cortical malformation syndromes (Baybis et al., 2004; Shaw et al., 2004; Kwiatkowski and Manning, 2005).

**Patients and Methods**

This study was approved by the Institutional Review Board of Lancaster General Hospital. Parents consented in writing to molecular testing and reproduction of photographs. Seven affected individuals and their 14 parents were used for SNP genome-wide autozygosity mapping (Strauss et al., 2005). After mapping the disease gene to chromosome 17, nine additional Mennonite children with suggestive clinical features were tested for the LYK5 deletion by direct molecular genetic analysis. Phenotype information was based on clinical data from 16 patients, ages 7 months to 28 years.

**Genotyping**

Total genomic DNA from whole blood was isolated using the PUREGENE DNA Isolation Kit (Genta Systems, Minneapolis, MN, USA) according to the manufacturer’s protocol. SNP genotyping was done using the GeneChip Mapping 10K Assay Kit (Affymetrix, Santa Clara, CA, USA) as previously described (Puffenberger et al., 2004). Briefly, 250 ng of double-stranded genomic DNA was digested with XbaI, the restriction fragments were ligated to adapters, and the ligated products were amplified in quadruplicate using a generic primer. PCR products were purified, fragmented with DNase, labelled with terminal deoxytransferase, and finally hybridized to a GeneChip Human Mapping 10K Xba142 2.0 Array. Microarrays were then washed with a fluids station, incubated and scanned using the GeneChip Scanner 3000 enabled by GeneChip GCOS software (Affymetrix).

**Genome-wide autozygosity mapping**

Data were analysed in Microsoft Excel spreadsheets (Microsoft Corporation, Redmond, WA, USA) that were custom-formatted at the Clinic for Special Children. SNP positions came from Affymetrix genome annotation files and genotype data came from the Affymetrix GeneChip Human Mapping 10K Xba142 2.0 Arrays. Excel spreadsheets were designed to identify genomic regions that were identical-by-descent between all affected individuals. This analysis assumes mutation and locus homozygosity. Two-point LOD scores were calculated for each genotype SNP using an approach similar to Broman and Weber (1999a, b). Cumulative two-point LOD scores for a block of homozygous SNPs were considered the location score for a region, providing a relative measure of likelihood that the region harbours the disease gene. Genotype data from this study and others (Puffenberger et al., 2004; Strauss et al., 2005, 2006) were used to estimate population-specific SNP allele frequencies.

**DNA sequencing**

The target sequence was amplified by using specific oligonucleotide primers and 30–50 ng of genomic DNA from affected and unaffected family members. Primer sequences for PCR amplification and sequencing were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR products were purified using QiaQuick columns (Qiagen, Valencia, CA, USA), as per manufacturer’s instructions, and then sequenced using the BigDye Terminator cycle sequencing protocol (Applied Biosystems, Foster City, CA, USA). Extension products were subsequently size-fractionated on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and data were compared to the human genome reference sequence and dbSNP in order to identify sequence variants. Population-based control samples were studied in an identical fashion.

**Genome-wide copy number analysis**

The SNP copy number generated by the copy number analysis tool (CNAT) of the Affymetrix Genotyping software was standardized for analyses. For each SNP on the array, we first generated a mean copy number and standard deviation from a reference population of 100 healthy (control) individuals from the Plain community. Copy number data for each patient were then exported to Excel files and SNP copy number z-scores (standard scores) were calculated according to the formula: $z = (\text{patient value} - \text{control mean}) / \text{control standard deviation}$. The resulting single-point copy number z-scores and log2 ratios were plotted using chart and trendline functions in Excel. Within a normal population, 99.8% of z-scores fall between −3 and +3; scores outside of these limits were investigated in more detail.

**Histology and immunohistochemistry**

An autopsy was performed on one female patient who died of an undetermined cause at 7 months of age. Paraaffin-embedded sections from liver, kidney, heart, lung and brain were stained with haematoxylin–eosin. Additional brain specimens were stained...
with luxol fast blue-cresyl violet (LFB-CV) and routine immunoperoxidase methods using antibodies against glial fibrillary acidic protein (GFAP). Sections of frontal cortex, striatum, hippocampus and spinal cord were probed with anti-phospho-ribosomal S6 (P-S6; Ser235/236; 1:50 dilution; Cell Signaling, New England Biolabs, Beverly, MA, USA) (Baybis et al., 2004), which were visualized with biotinylated anti-rabbit IgG (1:1000 dilution; Vector Laboratories, Burlingame, CA, USA) after avidin-biotin conjugation (Vectastain ABC Elite, Vector Laboratories) and treatment with 3,3′-diaminobenzidine.

Results

Clinical phenotype

Clinical data from 16 affected individuals (ages 7 months to 28 years) are summarized in Table 1. All affected pregnancies were complicated by polyhydramnios. Spontaneous onset of labour occurred between 25 and 36 weeks gestation in 12 pregnancies (75%). All but one affected child had macrocephaly (head circumference/C21 SD for age) (Fig. 1). Linear growth was normal and body mass index was consistently low (<13 kg/m²) due to low muscle mass. Several young children had persistent restless limb movements, a sign we interpreted as mild chorea. This was not a finding in older patients.

Table 1  Clinical features of 16 patients with homozygous LYK5 deletions

<table>
<thead>
<tr>
<th>Clinical variable</th>
<th>Percent of patients affected (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prenatal</strong></td>
<td></td>
</tr>
<tr>
<td>Polyhydramnios</td>
<td>100</td>
</tr>
<tr>
<td>Preterm laboura</td>
<td>75</td>
</tr>
<tr>
<td><strong>Postnatal</strong></td>
<td></td>
</tr>
<tr>
<td>Macrocephalyb</td>
<td>100</td>
</tr>
<tr>
<td>Infantile-onset partial epilepsy</td>
<td>100</td>
</tr>
<tr>
<td>Hypotonia</td>
<td>100</td>
</tr>
<tr>
<td>Craniofacial dysmorphism</td>
<td>100</td>
</tr>
<tr>
<td>Skeletal muscle hypoplasia</td>
<td>100</td>
</tr>
<tr>
<td>Strabismus</td>
<td>56</td>
</tr>
<tr>
<td>Atrial septal defect</td>
<td>25</td>
</tr>
<tr>
<td>Nephrocalcinosisc</td>
<td>13</td>
</tr>
<tr>
<td>Diabetes insipidisc</td>
<td>13</td>
</tr>
<tr>
<td>Supraventricular tachycardia</td>
<td>6</td>
</tr>
<tr>
<td>Leukaemia</td>
<td>6</td>
</tr>
<tr>
<td><strong>Developmental domainc</strong></td>
<td>Maximum developmental age (range in months)</td>
</tr>
<tr>
<td>Gross motor</td>
<td>6–14</td>
</tr>
<tr>
<td>Fine motor-adaptive</td>
<td>4–10</td>
</tr>
<tr>
<td>Language—communication</td>
<td>2–6</td>
</tr>
<tr>
<td>Personal—social</td>
<td>2–6</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| aMean onset of labour at 31 weeks gestation, range 25–37 weeks.  
| bDue to a combination of megalencephaly and hydrocephalus.  
| cRenal ultrasounds were only performed in four patients, two of whom (imaged at ages 6 and 28 years) had nephrocalcinosis.  
| dOne patient with DI had bilateral nephrocalcinosis on CT scan. The cause of DI was mixed, with both central and nephrogenic components.  
| eDevelopmental outcomes based on Denver Developmental Screening Test II. |

Each affected child had a long face, large forehead, peaked eyebrows, broad nasal bridge, hypertelorism and a large mouth with thick lips. Craniofacial structure changed considerably with age (Fig. 2A). During infancy and early childhood the skull was shaped like an inverted pyramid, reflecting prominence of the cranial vault relative to the lower face. As children grew older, their appearance changed due to overgrowth of the mandible, enlargement of the mouth and thickening of the lips (Fig. 2B). Radiological skeletal surveys, available for only two infants, were normal.

Cranial magnetic resonance imaging (MRI) studies at 1.5 Tesla were available from only four patients, ages 4 months to 5 years. These images were acquired 5 to 10 years ago, and thus the quality was poor relative to current imaging standards. The MRI from the two youngest children, whose head circumferences were greater than four standard deviations above normal, showed only mild ventriculomegaly that could not fully account for increased head size. Studies from the two older children showed ventriculomegaly, evidence of subependymal dysplasia and multiple areas of high water diffusion within the subcortical white matter (Fig. 3).

Seizures started between 3 and 7 months of age in all patients. These were most commonly complex partial seizures that would occasionally spread to involve one or both cerebral hemispheres. Electroencephalographic recordings typically showed high-voltage spike and slow wave discharges arising from bilateral independent electroencephalographic foci over the frontal, temporal, parietal and occipital regions. These spike-slow wave discharges would sometimes change into a long run of spikes, or into a rhythmic slow wave focus (2.5 to 3 cycles per second)
Fig. 2 Dysmorphic features. (A) Young persons with homozygous LYK5 deletions had a long face, large forehead, peaked eyebrows, broad nose and wide-set eyes. As patients got older (from upper left to lower right), the mouth became enlarged and the lips thickened. (B) Individual craniofacial structure changed considerably with age. Serial pictures of one affected individual show macrocephaly, frontal bossing, hypertelorism and broad nasal bridge shortly after birth (left panel), lengthening of the face during early childhood (middle panel), and overgrowth of the mandible, enlargement of the mouth and thickening of the lips during adolescence (right panel).
lasting 10 to 15 min. Two young patients were diagnosed with infantile spasms based on clinical and electroencephalographic criteria. One child had right-sided epilepsia partialis continua during a 24-h period of outpatient monitoring. In all patients, seizures were frequent and medically intractable during infancy and early childhood. Clusters of brief partial seizures, secondary seizure generalization and status epilepticus were common causes of hospitalization. Two young children died of seizure-related complications.

All patients had severe psychomotor retardation. Neurodevelopmental outcomes were uniformly poor. Projected adult mental ages were between 6 and 12 months (Table 1). Most of the affected individuals were confined to wheelchairs. Four patients learned to walk with assistive devices but subsequently regressed. All subjects were mute and fully dependent on others for feeding and self-care.

Four patients (25%) had atrial septal defects and one of these children developed congestive heart failure at 3 months of age. The atrial defects could not be classified (i.e., secundum, primum or sinus venosus) based on ultrasound reports. Diabetes insipidus was present in two patients that were formally tested and two additional patients had a clinical history suggestive of an osmoregulatory defect. Diabetes insipidus appeared to be of both central and nephrogenic origin. One 5-year-old subject had chronic polydipsia and polyuria and presented with recurrent episodes of hypernatremic dehydration. During one such episode (serum sodium 151 mEq/ml, serum osmolarity 327 mOsm/l), plasma arginine–vasopressin was inappropriately low (9.1 pg/ml) but urine was dilute (557 mOsm/l) relative to the arginine–vasopressin level. This patient and one other had bilateral nephrocalcinosis despite essentially normal urinary calcium levels (calcium/creatinine ratio 0.19–0.47 mg/mg; upper limit of normal for age 0.42).

Among 16 children with PMSE syndrome, six (38%) died between ages 7 months and 6 years. Causes of death were status epilepticus (n = 2), hypovolemic shock (secondary to diabetes insipidus) and leukaemia. Two young patients died suddenly and unexpectedly of undetermined causes.

**Gene mapping**

All seven children used for autozygosity mapping were Old Order Mennonite; four were from Lancaster County, Pennsylvania, two were from New York and one was from Iowa. They shared the same clinical phenotype and were related to one another, albeit distantly (Fig. 4A). Assuming mutation homogeneity, we searched SNP genotype profiles for homozygous regions shared among affected individuals. Analysis of the data with the GeneSpring GT software package (Agilent Technologies) yielded similar negative results.

In an effort to understand the inconclusive mapping results, we tested several alternative hypotheses for the
apparent lack of autozygosity, including mutation heterogeneity, locus heterogeneity and digenic inheritance. All of these tests were uninformative. We then concentrated on chromosomal regions with insufficient SNP coverage. While examining one such region on chromosome 17, we noticed a single SNP, rs721575, which produced ‘no calls’ for all seven patients (Fig. 5A). This suggested homozygous deletion of rs721575 in patients and hemizygosity of parents. Indeed, we found that all 14 parents of affected children were ‘homozygous’ for their respective rs721575 allele. This suggested that the marker was deleted in all patients and hemizygous in parents.

To further test this conclusion, we performed two separate analyses of the genotype data using the CNAT module of the Affymetrix software. The first analysis plotted log2 ratio data generated by the copy number tool (Fig. 5B), whereas the second calculated average copy number z-scores for each SNP from the seven affected patients. In the seven patients, the average z-score for rs721575 was $-4.16$; only one other SNP ($N=10,033$) fell outside the conservative cutoff range of 3 to $-3$. Results from both analyses were consistent with homozygous deletion of rs721575 in all affected children (Fig. 5).

We then proceeded to delineate the deletion boundaries. The Affymetrix protocol relies on genomic DNA digestion with the restriction endonuclease XbaI followed by amplification of restriction fragments in the 250–1000 bp size range. Amplified patient DNA is then labelled and hybridized to GeneChip arrays. Thus, the failure of
rs721575 to function in patients could have been due either to polymorphism within the flanking XbaI restriction sites or to polymorphic insertions or deletions between or involving the XbaI sites. To investigate these possibilities, we designed PCR primers to amplify rs721575 as well as the flanking XbaI sites. We were unable to generate PCR products using patient DNA. However, parent and control DNA produced appropriately sized products, which matched the normal human genome reference sequence and confirmed 'homozygosity' for rs721575 in all parents. These results indicated that patients were homozygous for a deletion encompassing both rs721575 and the flanking XbaI sites.

The SNP rs721575 lies within intron 10 of the LYK5 gene. PCR primers were generated for all LYK5 exons (isoform 1) and used to amplify patient samples. We successfully amplified LYK5 exons 1–8 in patients, but not exons 9–13. All 13 exons could be amplified from control DNA samples. Exon 17 of MAP3K3, distal to LYK5 and in an opposite orientation, was determined by PCR to be present in patient samples. Using the forward primer for exon 8 of LYK5, additional reverse primers were fashioned every 1 kb to delimit the size of the deletion. One primer set produced a product in patients, but not in controls. Sequencing of this product revealed a homozygous 7304 bp deletion that encompassed the terminal 5 exons of LYK5, but spared adjacent genes LOC440455, MGC10986 and MAP3K3 (Fig. 6).

Using primers designed to span the deletion breakpoints, we genotyped 100 healthy Old Order Mennonite controls. We identified four 7 kb deletion heterozygotes, yielding an estimated carrier frequency for the mutant allele in Lancaster County Old Order Mennonites of approximately 4%.

**Descriptive pathology**

A 7-month-old child with PMSE syndrome died unexpectedly following a prolonged cluster of seizures. At the time of death, she was 7 months old, 6.97 kg (−1.1 SD), 70 cm in length (+0.9 SD) and had a head circumference of 49 cm (+4.0 SD). Pathological examination did not reveal the cause of death. The visceral anatomy was normal, as was microscopic histology of lungs, myocardium, liver, ovaries, skeletal muscle, bone marrow, kidneys, thymus, pancreas, thyroid and parathyroid glands.

The fresh brain was 1134 g (normal ±95% CI for age 750 ± 184 g). The cerebral ventricles were enlarged and had patent outflow pathways. Leptomeningeal glioneuronal heterotopias were present at the ventrolateral surface of the pons and adjacent to the lateral geniculate body, but were not found over the neocortical surface.

Enlarged neurons with granular cytoplasm, diminished Nissl substance, areas of vacuolization and indistinct nuclei were found in the cerebellar Pukinje cell layer, hilus of the hippocampal dentate gyrus, trochlear (IV) nerve nuclei,
substantia nigra, anterior pituitary and anterior horn of the spinal cord (Fig. 7A and B). Large dysmorphic cells with similar characteristics were also seen in the frontal cortex, lentiform nuclei and pallidum (Fig. 8). Although neurons with these abnormal structural features were diffusely distributed, we did not find any definite abnormalities of cortical lamination or focal areas of dysplasia in the single brain specimen examined.

Small optically clear vacuoles were widely distributed throughout the white matter, and were most prominent at the neocortical and entorhinal gray matter–white matter transition zones (Fig. 7C and D). These vacuoles were organized in linear arrays parallel to, and often displacing, intact nerve fibres with normal appearing myelin. There was also marked vacuolization within the globus pallidus, head of the caudate, putaminal white matter bundles, external and extreme capsules, pontine tegmentum, ventral medulla and the dentate nuclei of the cerebellum (Fig. 7E and F). In contrast, corticospinal and pontocerebellar tracts appeared normal.

Astrocytes were abundant throughout the vacuolated white matter regions of the brain (Fig. 7C, D and F). There was also prominent astrogliosis in the hippocampal formation, particularly within the hilus and adjacent to the dentate granule-cell layer (Fig. 7G and H). Astrocyte processes were often in physical contact with fluid vacuoles (Fig. 7D, inset) or neuronal cell bodies (Fig. 8D) and some astrocytes were large and in the process of duplication (Fig. 7G, inset).

Labelling of mTOR cascade components
There was a low-level of P-S6 expression in control brain tissue. In the brain of a child with PMSE syndrome, P-S6 expression was increased throughout all six layers of neocortex, and was most prominent in pyramidal cells of layer V. Within the entorhinal cortex, there was robust P-S6 expression within islands of large pyramidal cells of layer II. In the hippocampus, large dysmorphic P-S6-labelled neurons were present in the hilus of the dentate gyrus, and there was modest P-S6 expression in CA sectors. There was robust P-S6 expression in large neurons of the anterior horn of the spinal cord, cerebellar Purkinje cell layer and basal ganglia (Fig. 8C), and P-S6 was evident in many cranial motor nuclei (e.g. III, VI and VII) of the brainstem.

As only a limited amount of tissue from a single brain was available for pathological inspection, additional analyses of gene and protein expression were not possible.

Discussion
The use of microarrays for investigating novel clinical phenotypes
Since 1998, we have identified molecular lesions for more than 65 monogenic disorders segregating in Plain populations (Morton et al., 2003). Developmental delay is the presenting problem for 29 (45%) of these, 10 of which are associated with distinctive physical features. Nevertheless, evaluating a child with syndromic developmental delay remains a difficult diagnostic problem. For such patients, we increasingly rely upon small-scale microarray studies to provide focus and direction (Puffenberger et al., 2004; Strauss et al., 2005, 2006). Indeed, SNP genotyping is often the first step of the diagnostic process. When coupled to population-based genetic knowledge (Morton et al., 2003; Puffenberger, 2003), it is an efficient way to detect DNA copy number abnormalities, scan candidate regions for homozygosity, and determine shared haplotypes among as few as two patients with a similar phenotype.

Among the present group of patients, there were no clues from the clinical or MRI data to implicate LYK5 deficiency, and no analyte testing could have uncovered the diagnosis. Thus, a small-scale mapping study was the only conceivable way to investigate the problem. Because we work with a young, genetically isolated population (Puffenberger, 2003; Puffenberger et al., 2004; Strauss et al., 2005, 2006), we expected autozygosity mapping would be straightforward.

Our initial results (Fig. 4B) highlight important pitfalls of small mapping studies. The lack of significant homozygous blocks could have resulted from mutation or locus heterogeneity. Identity-by-descent analyses assume mutation and locus homogeneity; however, even in small isolated populations such as the Amish and Mennonites, heterogeneity does exist (Puffenberger, 2003). For this study, further statistical analyses were necessary to show that mutation and locus heterogeneity were not present.

A dearth of SNPs in the disease gene region was the primary reason that autozygosity mapping failed to reveal a large shared homozygous block. Although most genomic regions are well represented on the Mapping 10K Array, coverage is uneven and significant gaps exist. LYK5 lies in one of those gaps. The flanking SNPs, rs1960286 and rs230572, are 3.845 Mb apart (Fig. 5A). For comparison, our mapping study of SIDDT syndrome identified a 3.6 Mb homozygous region shared among four Amish patients (Puffenberger et al., 2004). If the region surrounding LYK5 had the average SNP coverage ~3 SNPs per Mb (for the Mapping 10K Array), then identifying the region by autozygosity mapping would have been simpler, as we have shown previously (Puffenberger et al., 2004; Strauss et al., 2005, 2006). For this study, use of a higher density array would have likely solved this problem.

Fortunately, the single SNP included on the Mapping 10K Array that localized to the 4 Mb region between rs1960286 and rs230572 happened to lie within the deleted region of LYK5. Although not explored in our earlier studies, we show here that the homozygous deletion of a single SNP is easily detected when appropriate analyses are employed. Average ‘no call’ rates for individual SNPs in a reference population provided an important first clue to identification of the molecular lesion in our patients. Formal assessment of copy number changes using the
Fig. 7  Histological findings in the brain of a 7-month-old infant who had PMSE syndrome. (A) In the anterior spinal cord, motor neurons are enlarged, with rounded cell bodies, granular cytoplasm and an indistinct nuclear membrane. Neurons with similar morphology were found in select areas of the brainstem, cerebellum, basal ganglia, anterior pituitary and cortex (LFB-CV, 600×). For comparison, panel B shows normal appearing anterior horn cells from a control spinal cord specimen that was stained in parallel and viewed at similar magnification. (C) Astrocytes are abundant within the vacuolated gray matter–white matter transition zone (dotted line) of the frontal cortex (GFAP, 20×). (D) A higher magnification view (100×) shows optically clear vacuoles in linear arrays, often in direct contact with astrocytes (arrow, inset). (E and F) Vacuolization and astrocytosis in the dentate nucleus of the cerebellum (40×; panel E = LFB-CV; panel F = GFAP). (G) There is astocytosis in the hippocampal formation, particularly within the hilus and adjacent to the dentate granule-cell layer (dotted line) (GFAP, 40×). Some astrocytes are in the process of duplication (arrow, inset). Panel H is a magnified view of hippocampal dentate granule cells with adjacent astrocytes (GFAP, 400×).
Affymetrix Copy Number Analysis Tool (CNAT) as well as the calculation of standard scores (z-scores) was indispensable for identification of the LYK5 deletion. Given the increasing density of SNP microarrays, we anticipate that copy number analysis will become a routine part of disease gene mapping. The same set of SNP genotypes can be used not only to identify autozygous genomic regions, but also to detect loss of alleles, even for a single SNP.

**Biological relevance of the PMSE syndrome**

The LYK5 gene product, STRAD, is expressed in human embryonic specimens and a wide variety of mature tissues, including kidney, muscle, peripheral nerve and brain (http://cgap.nci.nih.gov). It is part of a trimeric complex with serine–threonine kinase 11 (STK11, a.k.a. LKB1) and MO25 (a.k.a. calcium binding protein 39; CAB39) (Fig. 9) (Boudeau et al., 2003; Baas et al., 2004b). LYK5 is necessary for normal cytosolic localization and kinase activity of STK11 (Boudeau et al., 2003).

STK11-STRAD-MO25 functions primarily as a tumour suppressor (Baas et al., 2003, 2004a, b). Haploinsufficiency of STK11 is associated with the Peutz–Jeghers syndrome, which is characterized by the development of multiple gastrointestinal hamartomatous polyps and a wide spectrum of benign and malignant tumors (Hemminki, 1999). While one affected child from our series died of leukaemia (Table 1), we cannot prove that it was causally related to the LYK5 mutation, and other tumours were not evident among patients. Our cohort was young (median age 16 years, range 7 months to 28 years), however, and a predisposition to tumours may turn out to be a later manifestation of PMSE syndrome. Moreover, none of our patients had colonoscopies, and polyposis would have gone unrecognized.

The clinical manifestations of PMSE syndrome could not be anticipated from prior *in vitro* studies of STRAD protein distribution and function (Hemminki, 1999; Milburn et al., 2004; de Leng et al., 2005). The phenotype associated with LYK5 deletions in humans (Table 1) shows that STRAD has a key role in shaping the nervous system, and may also influence cerebrospinal fluid transport, skeletal muscle growth, cardiac septation and renal physiology. Our findings underscore the fact that data about protein

---

**Fig. 8** P-S6 immunohistochemistry and cytomegaly in the putamen of a child with PMSE syndrome. (A) Numerous large cells (arrows) are seen with a LFB-CV at 100×. (B) At higher magnification (LFB-CV, 400×), these cells are rounded, have granular cytoplasm, loss of Nissl substance, and indistinct nuclei and nucleoli; they are structurally similar to the anterior horn cells depicted in Fig. 7A. (C) Large cells appear to have neuronal morphology and are strongly immunoreactive for P-S6 (inset). Similar anti-P-S6 reactivity was seen in large neurons of the caudate nucleus, hippocampus and anterior horn of the spinal cord. (D) GFAP immunostaining shows intense vacuolization and astrogliosis around these large neurons (asterisks) (400×).
function derived from animal knockout and *in vitro* studies may be misleading when extrapolated to human biology (Strauss et al., 2006).

**LYK5 and mTOR activation**

In one post-mortem case available for analysis, we found evidence of megalencephaly, cytomegaly, astrocytic gliosis and white matter vacuolization (Figs 7 and 8). A focal cortical dysplasia was not available to examine from this single brain specimen, but limited MRI evidence shows that such lesions may be associated with homozygous **LYK5** deletions in some patients (Fig. 3). Thus, although the histological observations should be interpreted cautiously, they suggest that this condition is a new syndromic malformation of cortical development. Further histopathological studies of post-mortem or surgical tissue are necessary to define the exact nature and extent of cortical dysplasia associated with **LYK5** mutations. Magnetic resonance imaging at 1.5 Tesla does not have sufficient resolution for this purpose (Strauss et al., 2006).

Interestingly, the widespread cytomegalic cells expressing P-S6 in the brain of an infant with PMSE syndrome were similar to the giant cells of tuberous sclerosis complex and the balloon cells of focal cortical dysplasia type II (Baybis et al., 2004; Miyata et al., 2004). This observation suggests that loss of **LYK5** function during brain development may lead to downstream activation of the mTOR cascade. Indeed, in normal cells **LYK5** (as part of trimeric complex with STK11 and MO25) participates in the phosphorylation of AMPK, which in turn phosphorylates the TSC2 protein at Ser1345, thereby inhibiting mTOR (Fig. 9).

We postulate that mTOR is constitutively activated as a result of homozygous **LYK5** mutations, and that this leads to inappropriate phosphorylation of p70 S6 kinase and P-S6 (Fig. 9). Thus, the cellular mechanisms leading to abnormal brain development from **LYK5** deletions may be similar to those in TSC. Quantitative analysis of protein expression (i.e. reduced phospho-AMPK or enhanced phospho-mTOR) was not feasible because frozen brain tissue was not available; additional studies of both human tissue and experimental animals are necessary to fully explore this hypothesis. Here, we show that P-S6 expression was increased in large dysmorphic neurons throughout the neuraxis. We speculate that high P-S6 expression in various brain regions may be associated with abnormal cellular functions that manifest as severe cognitive disabilities and epilepsy in all affected children, and movement disorders in some younger patients.

Widespread white matter vacuolization and astrocytosis were the most prominent histological features of the single brain we studied (Fig. 7C–F). Increased numbers of astrocytes are also found in cortical tubers (Scheithauer and Regan, 1999), and abnormal white matter structure has been described in TSC brains (Ridler et al., 2001). In both TSC and PMSE syndrome, proliferation of astrocytes may result from constitutive activation of mTOR (Fig. 9), either focally (TSC) or diffusely (PMSE). Alternatively, gliosis may simply be a reactive process in response to abnormal neuronal structure and activity. The mechanism of white matter vacuolization is unknown.
Conclusions and future directions

We used the LYK5 mutation information to develop a rapid, inexpensive molecular diagnostic test. This test will spare children and families protracted, costly and uninformative diagnostic evaluations (Strauss et al., 2005). In practice, this will allow us to anticipate problems like heart failure, hyponatremic dehydration and status epilepticus. These are serious clinical conditions for which prevention, not symptomatic treatment, is the goal (Morton et al., 2003).

The identification of LYK5 deletions in affected patients is a starting point for considerations about treatment, which may, for example, focus on mTOR or related signalling pathways (Fig. 9) (Kenerson et al., 2005; Kwiatkowski and Manning, 2005; Kesari et al., 2006; Doherty et al., 2006). The success of such therapies will depend on diagnosing the condition early in life, before intractable focal seizures injure the developing brain (Holmes and Ben-Ari, 1998, 2001).

Disclosure Statement

The authors declare that the Affymetrix GeneChip Scanner used for a portion of this study was a charitable donation to the non-profit Clinic for Special Children (EIN 23-2555373) from the Affymetrix Corporation.

Supplementary material

Supplementary material is available at BRAIN online.

Acknowledgements

The authors dedicate this paper to all of the families who cooperated to make this work possible. The authors extend a special thank you to Mark and Dorothy Weaver, for initially identifying the seven LYK5 deletion patients used for mapping, and organizing the families to participate in this study. We are grateful to Christine Weir and the technical staff of the Lancaster MRI group, as well as Rebecca LeVier and her colleagues in the Department of Pathology, Lancaster General Hospital. This study was supported in part by grant U24NS051872 from the National Institutes of Health to Dietrich A. Stephan.

References


Downloaded from https://academic.oup.com/brain/article-abstract/130/7/1929/325592 by guest on 16 March 2019