RARE GENETIC CAUSES OF MICROCEPHALY AND MEGALENCEPHALY

Ph.D. Thesis

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PUBLICATIONS RELATED TO THE THESIS

1. **Zombor M**, Kalmár T, Nagy N, Berényi M, Telcs B, Maróti Z, Brandau O, Sztriha L. A novel *WDR62* missense mutation in microcephaly with abnormal cortical architecture and review of the literature. J Appl Genet 2019;60:151-162. (**Impact factor: 1.725**).
2. **Zombor M**, Kalmár T, Maróti Z, Zimmermann A, Máté A, Bereczki Cs, Sztriha L. Co-occurrence of mutations in *FOXP1* and *PTCH1* in a girl with extreme megalencephaly, callosal dysgenesis and profound intellectual disability. J Hum Genet 2018;63:1189-1193. (**IF:3.545**)
3. Alcantara D, Timms AE, Gripp K, Baker L, Park K, Collins S, Cheng C, Stewart F, Mehta SG, Saggar A, Sztriha L, **Zombor M,** Caluseriu O, Mesterman R, Van Allen MI, Jacquinet A, Ygberg S, Bernstein JA, Wenger AM, Guturu H, Bejerano G, Gomez-Ospina N, Lehman A, Alfei E, Pantaleoni C, Conti V, Guerrini R, Moog U, Graham JM. Hevner R, Dobyns WB, O'Driscoll M, Mirzaa GM. Mutations of *AKT3* are associated with a wide spectrum of developmental disorders including extreme megalencephaly. *Brain* 2017;40:2610-2622. (**IF:10.840**).

INTRODUCTION

Microcephaly has been defined as an occipitofrontal head circumference of equal to or less than 2-3 standard deviations (SD) below the mean related for age and gender. Microcephaly may develop prenatally (congenital microcephaly) or postnatally and may have genetic or non-genetic causes. The spectrum of phenotypes and associated disorders of "microcephaly" is wide with 1429 entries recorded to September 2019 in The Online Mendelian Inheritance in Man (OMIM) database.

Although a wide spectrum of genetic defects can result in microcephaly, traditionally a group of microcephalies is distinguished as autosomal recessive primary microcephaly (MicroCephaly Primary Hereditary, MCPH). At least 18 genes (MCPH1-18 until September 2019) have been implicated in MCPH. Many of the proteins encoded by these genes interact with the centrosomes, which organizes the separation of chromosome copies during cell division. In addition to simplified gyri, a wide spectrum of cortical malformations have been revealed in association with MCPH of different aetiologies, particularly *WDR62* mutations.

We report on three patients who had autosomal recessive primary microcephaly in association with abnormal cortical architecture: a boy with a novel homozygous nonsense mutation in the *ASPM* gene and two other patients, a boy and a girl with the same novel homozygous missense mutations in *WDR62*.

Megalencephaly and **macrocephaly** have been defined as conditions with head circumference that exceeds the mean by 2 or more standard deviations (SD) for age and gender. Megalencephaly defines an increased growth of cerebral structures because of either increased size or number of neurons and glial cells. In contrast, in macrocephaly, the increased head circumference is linked to hydrocephalus, subdural fluid collections, or intracranial masses.

Megalencephaly can be *idiopathic/benign* or it can be associated with *metabolic disorders*, or *genetic syndromes*. The phenotypic features and molecular genetic findings of two patients with megalencephaly are presented in this study. A girl with extreme megalencephaly and profound intellectual disability had *de novo* heterozygous mutations in two genes, such as *FOXP1* and *PTCH1*. Megalencephaly, diffuse polymicrogyria and delayed development were the characteristic features of a boy with a germline *de novo* heterozygous mutation in *AKT3*.

Microcephaly and megalencephaly of genetic aetiology are rare conditions; hence, it is justified to devote this study (Ph.D. Theses) to the thorough work up of these

patients. It is in line with the international endeavour to identify the genetic causes of rare diseases by applying new technologies, particularly array methods and next generation sequencing.

OBJECTIVES

The aims of this study were:

1. / to ascertain patients with rare forms of *autosomal recessive primary microcephaly* and *megalencephaly*
2. / to assess these patients, including physical and neurological examination and developmental testing
3. / to evaluate the brain MR images
4. / to identify the molecular genetic causes of these conditions

METHODS

Patients with autosomal recessive primary microcephaly (3 children) and megalencephaly (2 children) were enrolled out of the patient population referred to the Paediatric Neurology Service (outpatient and inpatient), Department of Paediatrics, Division B, University of Szeged, Hungary between 1 January 2006 and 31 December 2017.

Standard assessment included detailed clinical history taking followed by physical and neurological examinations. Special attention has been paid to the presence of dysmorphic features and malformations. Hearing was always tested and neuro-ophthalmologic consultation was also an integral part of the examinations. The developmental milestones, cognitive and behavioural phenotypes were also assessed. Screening for inborn errors of metabolism and intrauterine infections were carried out by conventional methods. Brain MRI was performed in axial, coronal and sagittal planes.

Routine chromosomal analysis was carried out by G-banding. Genomic DNA was isolated from the peripheral blood in all patients and saliva as well in Patient 5. Array comparative genomic hybridization was performed using the Affymetrix 750K array.

In **Patients 1-3** whole exome sequencing (WES) was performed with CentoXome® at Centogene AG (Rostock, Germany). Genomic capture was carried out with Illumina's Nextera Rapid Capture Exome Kit. Massively parallel sequencing was done using NextSeq500 Sequencer (Illumina) in combination with the NextSeq™ 500 High Output Kit (2x150bp). Raw sequence data analysis, including base calling, de-

multiplexing, alignment to the hg19 human reference genome (Genome Reference Consortium GRCh37), and variant calling, were performed using an in house bioinformatics pipeline. For variant filtration, all disease-causing variants reported in Human Gene Mutation Database (HGMD®), ClinVar or in CentoMD® as well as all variants with minor allele frequency (MAF) of less than 1% in Exome Aggregation Consortium (ExAc) database were considered. Variants that possibly impair the protein sequence, i.e. disruption of conserved splice sites, missense, nonsense, read-throughs, or small insertions/deletions, were prioritized. All relevant inheritance patterns were considered.

In **Patient 4** the Illumina Trusight One Exome Sequencing Panel (Illumina Inc., San Diego, CA, USA), covering the coding region of 4,813 clinically relevant genes, was applied using Illumina MiSeq (Illumina Inc., San Diego, California, USA). Variants were filtered based on severity and frequency against public variant databases including single-nucleotide polymorphism database (dbSNP), ClinVar, ExAC, Exome Variant Server (EVS) and in-house clinical exome database of 140 unrelated Hungarian persons. All relevant inheritance patterns were considered.

In **Patient 5** multiplex targeted sequencing was applied using single molecule molecular inversion probes.

All candidate pathogenic variants were confirmed by conventional PCR amplification and Sanger sequencing. Segregation of these changes with the disease was assessed for all available family members.

Haplotype analysis of the families

Since the families of **Patients 2-3** with the same *WDR62* mutation were unaware of any relation between them, we performed a haplotype analysis to investigate their potential genetic relation. Plink (version v1.90b4.9) was used to convert variants in the region of interest to Pedigree (PED) and MAP files from the joint Variant Call Format (VCF) file. Haplotype analysis was performed by Merlin (version 1.1.2.) software with the "--best" option using the PED, Data (DAT), and MAP files prepared manually from the plink output files. HaploPainter (version 1.043) was used to visualize the haplotypes in the families.

Informed consent to participate in this study was requested from the parents. This study was approved by the *Human Investigation Review Board, University of Szeged, Albert Szent-Györgyi Clinical Center (3797/2016.*

PATIENTS AND RESULTS

Table Genes and mutations reported in this study

Patients	Genes	Exons	Nucleotide variation	Amino acid variation	Mutation type	Zygosity	Main phenotypic feature	Comments
1	*ASPM*	Exon 18	c.7323T>A	p.Tyr2441Ter	Nonsense	Homozygous	Microcephaly	Novel
2	*WDR62*	Exon 6	c.668T>C	p.Phe223Ser	Missense	Homozygous	Microcephaly	Novel
3	*WDR62*	Exon 6	c.668T>C	p.Phe223Ser	Missense	Homozygous	Microcephaly	Novel
4	*FOXP1* *PTCH1*	Exon 18 Exon 17	c.1573C>T c.2834delG insAGATGTTG TGGACCC	p.Arg525Ter p.Arg945Glnfs Ter22	Nonsense Frameshift	Heterozygous *de novo* Heterozygous *de novo*	Megalencephaly	Published Novel
5	*AKT3*	Exon 13	c.1393C>T	p.Arg465Trp	Missense	Heterozygous *de novo*	Megalencephaly	Published

MICROCEPHALY (PATIENTS 1-3)

Patient 1 - *ASPM* mutation

This boy was born from the first pregnancy. His birth weight was 2350 g (-0.9 SD) at the 36th gestational week, length 47 cm (-0.1 SD) and head circumference ~30 cm (-1.8 SD). The microcephaly progressed with head circumference of 39 cm (-5.1 SD) at 10 months, 40.5 cm (-5.2 SD) at 18 months, and 43.5 cm (-5.7 SD) at 5.5 years of age. Motor and intellectual development was severely impaired with inability to sit and stand unsupported or speak at the age of 5 years. Typical signs of spastic tetraplegia (spastic quadriplegia) were observed. Tonic-clonic seizures appeared at the age of 18 months and they were well controlled with valproate. Brain MRI at age of 7 months showed an abnormal cortical pattern with features of cortical dysplasia and polymicrogyria. The grey-white matter junction appeared indistinct at some areas.

WES trio (Centogene AG) identified a novel homozygous pathogenic variant, c.7323T>A, p.(Tyr2441Ter) in exon 18 of the *ASPM* gene in the patient. This variant created a premature stop codon and it was consistent with the diagnosis of autosomal recessive primary microcephaly type 5. The detected variant was also found in heterozygous state in the patient's parents.

Patient 2 – *WDR62* mutation

This boy was born at term from the third pregnancy with Caesarean section to healthy consanguineous parents of Romani ethnicity. Severe microcephaly was noted at birth with head circumference of 30 cm (-3.5 SD). The birthweight was 2900 g (-1.0 SD) and length 50 cm (0.1 SD). The parents have a healthy son and a healthy daughter. Microcephaly progressed, with head circumference of 40 cm (-6.0 SD) at 2 years and 41.5 cm (-6.0 SD) at 4 years of age. The patient also had short stature, with a height of 89 cm (-3.4 SD) and a weight of 12 kg (-2.4 SD) at 4 years of age. His motor and intellectual development was severely impaired with inability to sit and stand unsupported, or reach out for objects at the age of 5 years. Generalized hypotonia was present with preserved deep tendon reflexes. Infantile spasms began at 4 months of age, followed by complex partial seizures responding tom vigabatrin and valproate treatment. MRI at age of 5 months showed hemispherical asymmetry (R>L) and abnormal cortical pattern. Diffuse pachygyria was observed with a few broad gyri, thick grey matter and shallow sulci.

Patient 3 – *WDR62* mutation

This girl was born at term from the first pregnancy with Caesarean section to healthy parents of Romani ethnicity. They denied consanguinity. Severe microcephaly

was noted at birth with head circumference of 28 cm (-5.0 SD). Her birthweight was 2490 g (-0.4 SD) and length 46 cm (-1.7 SD). Microcephaly progressed, with head circumference of 39 cm (-5.9 SD) at 2 years and 40 cm (-6.6 SD) at 4 years of age. The patient also had short stature, with height of 88 cm (-3.4 SD) and weight of 12,4 kg (-2.0 SD) at 4 years of age. Her motor and cognitive development was severely delayed with inability to sit, stand or reach out for objects. Complex partial seizures started after 3 years of age, which were well controlled with valproate. MRI at the age of 4 years showed hemispherical asymmetry (L>R) and abnormal cortical pattern similar to Patient 2.

WES trio (Centogene AG) identified the same novel homozygous missense variant, c.668T>C, p.(Phe223Ser) in exon 6 in the *WDR62* gene in Patients 2 and 3. The detected variant was also found in heterozygous state in the patients' parents and the brother of Patient 1. The family members of Patients 2 and 3 were unaware of any relatedness. Haplotype analysis showed that both families carry exactly the same haplotype for the entire *WDR62* gene (around 55 kilobases), suggesting that the two families are closely related.

MEGALENCEPHALY (PATIENTS 4-5)

Patient 4 – Coexistence of *FOXP1* and *PTCH1* mutation

The proband, a girl was born as the second child to healthy, non-consanguineous parents at 36 weeks of gestation with a birth weight of 2,870 g (0.4 SD), length of 50 cm (0.8 SD) and head circumference of 37 cm (1.8 SD).

In addition to several dysmorphic features, progressive growth of her head circumference was noted; it was 58 cm (6.1 SD) at the age of 4 years, and 61 cm (7.1 SD) at the age of 8 years. Her height was 129 cm (0.2 SD) and weight 33 kg (1.3 SD) at this age. Generalized hypotonia, unstable gait with preserved deep tendon reflexes were observed. Her motor and cognitive development was severely delayed with inability to speak or follow commands at the age of 8 years. Brain MRI, performed at 3 months, 3 and 4 years of age revealed partial agenesis of the corpus callosum and widely separated leaves of the septum pellucidum.

Clinical exome (Trusight One panel) sequencing revealed *de novo* heterozygous variants in exon 18 of the *FOXP1* [c.1573C>T, p.(Arg525Ter)] and exon 17 of the *PTCH1* [c.2834delGinsAGATGTTGTGGACCC, p.(Arg945GlnfsTer22)] genes in the patient. The *FOXP1* variant has been reported earlier as a nonsense pathogen mutation,

while the *PTCH1* variant has not been found in either the databases, or a cohort of 140 unrelated Hungarian controls. None of these mutations were present in the parents or in the patient's healthy brother.

Patient 5 - *AKT3* mutation

The patient, a boy was born by Caesarean section from the second uneventful pregnancy at 36th gestational weeks to unrelated healthy Caucasian parents. His birthweight was 3280g (1.3 SD), length 50 cm (1.2 SD) and head circumference 37.5 cm (3.3 SD). He had a healthy brother. His head circumference was 51 cm (5.1 SD) at 7 months of corrected age, 53 cm (4.5 SD) at 15 months and 55 cm (4.1 SD) at 28 months of age. His weight was 11.5 kg (-1.3 SD) and height 82 cm (-2.2 SD) at this age. Delayed motor and intellectual development was noticed. Recurrent tonic-clonic seizures, well controlled with levetiracetam appeared after the age of 11 months. MRI was at 7 months of age revealed polymicrogyria in the perisylvian, frontal, parietal and temporal areas on both sides.

Targeted next generation sequencing revealed a heterozygous missense variant [c.1393C>T, p.(Arg465Trp)] in exon 13 of the *AKT3* gene. This pathogenic variant was present in 50% of reads in the DNA samples derived from both the blood and saliva, suggesting a constitutional mutation. It was *de novo*, absent in the parental samples.

DISCUSSION

MICROCEPHALY

Proliferation of the neural progenitor cells

Brain size at birth is primarily dependent on the ability of neural progenitor cells (neuroepithelial cells, radial glia cells and short neural precursors with apical-basal polarity lining the ventricles of the foetal brain) to proliferate and self-renew. While symmetrical division of a neural progenitor cell results in the generation of two identical neural progenitor cells (thereby increasing the progenitor pool), asymmetrical division leads to the production of one progenitor cell (thereby maintaining the progenitor pool) and a committed precursor, which eventually undergoes migration and differentiates into neurons. Centrosomes play an essential role in cell division, as they are responsible for the formation and maintenance of the microtubule-based spindle apparatus.

ASPM and _WDR62_ encode centrosomal proteins functioning in the division of neural progenitor cells

ASPM (abnormal spindle-like, microcephaly-associated protein) mutations cause MCPH5 (MicroCephaly Primary Hereditary 5), while *WDR62* (WD repeat containing protein 62*)* mutations are responsible for MCPH2. These are the most common causes of autosomal recessive primary microcephaly. *ASPM* maps at 1q31.3 locus and encodes a spindle pole associated protein. More than 200 mutations, mostly nonsense or frameshift mutations, randomly distributed over the gene have been published so far. No genotype-phenotype correlation has been found. In our patient the nonsense *ASPM* mutation [c.7323T>A, p.(Tyr2441Ter)] in exon 18 creating a premature stop codon may result in either truncation of the protein, or unstable mRNA that would be degraded by nonsense–mediated RNA decay.

Simplified gyral pattern, corpus callosum anomalies, cerebellar and/or pontine hypoplasia have been reported as the most frequent cerebral anomalies in *ASPM*-related microcephaly. In contrast to these findings, our patient showed very severe cortical dysplasia with features of polymicrogyria. A single patient with similar malformation and another one with unilateral polymicrogyria have been published in the literature so far.

The human ***WDR62*** gene maps to chromosome 19q13.12, and encodes a protein containing several WD40 repeats. More than 30 pathogenic mutations in *WDR62* have already been published. The frameshifts, missense, nonsense and splice site mutations in the *WDR62* gene are randomly distributed. In addition to microcephaly, a wide range of malformations, including pachygyria, cortical thickening, cortical dysplasia, heterotopia, polymicrogyria, schizencephaly, dysmorphic hippocampus, corpus callosum abnormalities and cerebellar hypoplasia was described in these patients.

We found diffuse pachygyria, thickened cortex and indistinct grey-white matter junction in our two patients from related families with the same missense *WDR62* mutation [c.668T>C, p.(Phe223Ser)]. This mutation affects one of the WD40 repeat regions of the WDR62 protein. WD40 repeat is a short structural motif of approximately 40 amino acids. The common function of all WD40 repeat proteins is coordinating multiprotein complex assemblies, where the repeating units serve as a rigid scaffold for protein interactions.

Experimental data on _Aspm_

In embryonic mice Aspm protein was highly expressed in the neuroepithelial cells when symmetric, proliferative divisions prevailed and declined progressively at later

stages of neurogenesis. It was recruited to the pericentriolar matrix at the spindle pole and exerted a critical role at the spindle poles of neuroepithelial cells in maintaining spindle axis exactly perpendicular to the neuroepithelial cell apical-basal axis throughout mitosis. It ensured the precise cleavage plane orientation required for symmetric, proliferative divisions. Loss of Aspm in knockdown mice resulted in deviation of spindle position, hence increasing the probability of asymmetric division of neuroepithelial cells, causing reduced expansion of neuroepithelial pool and leading to primary microcephaly.

Recent investigations revealed that ASPM has a mitotic orientation-independent effect on cell cycle duration. It has been shown in *Aspm* mutant mice that the cell cycle of the neural progenitor cells was prolonged, presumably promoting asymmetric division of the neural progenitor cells.

Experimental data on *Wdr62*

In vitro tests on cells with *WDR62/Wdr62* mutations and *in vivo* experiments in mice with knockdown or genetic inactivation of *Wdr62* showed similarities to the abnormalities found in patients with *ASPM* mutations and mice with *Aspm* knockdown. The regulation and subcellular localization of WDR62 was found to be cell cycle dependent. WDR62 protein accumulated strongly at the spindle poles during mitosis and showed cytosolic distribution in the interphase. Fibroblasts from a patient with homozygous *WDR62* mutation, or cells transfected with missense and frameshift *WDR62* mutations failed to show protein expression at the spindle poles. *In vivo* experiments in mice with *Wdr62* mutations showed impaired proliferation of neocortical progenitors, reduced cortical thickness and small brain. Abnormalities in the centriole duplication, spindle pole orientation, symmetric/asymmetric division of neural progenitor cells, defects in the mitotic progression, and premature delamination were also noticed in addition to increased apoptosis.

Experimental data on Aspm and Wdr62 interaction

There is a physical interaction between Aspm/ASPM and Wdr62/WDR62 proteins. It has been shown that Wdr62 is required to localize Aspm and other microcephaly–associated proteins to the centrosome. Mouse embryonic fibroblasts deficient in *Aspm, Wdr62*, or both show centriole duplication defects. It seems likely that centriole number and organization are critical for progenitor attachment to the ventricular surface and the maintenance of neural progenitors. Taken together, the experimental data provided some insight into the role of Aspm/ASPM and Wdr62/WDR62 function in the

development of brain size, however failed to clarify the precise mechanism of the wide range of structural abnormalities.

MEGALENCEPHALY

FOXP1 gene

FOXP1 (forkhead box protein P1) belongs to the functionally diverse family of forkhead (FOX) transcription factor proteins and acts as a transcriptional repressor. The *FOXP1* gene is located on chromosome 3p13. Monogenic *FOXP1* pathogenic variants (deletions, missense, nonsense, frameshifts and splice site mutations), or more extensive 3p chromosomal deletions encompassing *FOXP1* have been reported previously in approximately 50 patients. No specific genotype-phenotype correlation was found. A *FOXP1* mutation-related phenotype as a recognizable entity has been outlined with intellectual disability, specific language impairment with or without autistic spectrum disorder and dysmorphic features. The c.1573C>T, p.(Arg525Ter) heterozygous nonsense *de novo FOXP1* mutation in our patient causes premature truncation of the protein in the evolutionally conserved DNA-binding domain by abolishing the last 152 amino acids of FOXP1. *In vitro* functional characterization of *FOXP1* variants provided evidence that this mutation was pathogenic. Direct fluorescence imaging studies showed that the wild type protein was localized to the nuclei, excluded from the nucleoli, while the p.Arg525Ter variant was excluded from the nuclei and formed large cytoplasmic aggregate suggesting misfolding of the aberrant protein. In addition, the variant lost its transcriptional repressive activity pointing to disturbance of transcription regulation. FOXP proteins regulate gene expression by forming homo- and hetero-dimers with each other and they interact with other transcription factors forming a network involved in cortical development. Protein interaction studies revealed that the p.Arg525Ter variant did not interact with wild type FOXP1/FOXP2 and was unable to self-associate, likely because of misfolding.

Experimental data on *FOXP1*

The precise functions of FOXP1 transcription factor in brain development remained unclear so far. Foxp1 (FOXP1 in humans) was found to be expressed in the developing cerebral cortex in humans and experimental animals following neuronal migration suggesting a role for this protein in neuronal differentiation. Brain specific homozygous *Foxp1* deletion resulted in postnatal reduction of the dorsal and enlargement of the ventral regions of the striatum in association with developmental delay and

impaired behaviour. It is noteworthy, however, that an increase in the total brain volume, or abnormalities of the corpus callosum were not described in these mutant mice. Recently, neuronal migration defect with ectopic neurons in deep layers of the cortex have been found in mice following down-regulation of *Foxp1* expression in the cerebral cortex. Migration, or cortical lamination defect, however, were not seen in our patient by conventional MRI.

***PTCH1* gene**

The *PTCH1* gene on chromosome 9q22.3 is a human homolog of the Drosophila segment polarity gene, patched (*patched homolog 1, ptc,* or *Patched1* in some of the publications on rodents). It encodes a transmembrane glycoprotein with 12 transmembrane regions, an intracellular and two extracellular loops, and a putative sterol-sensing domain. It functions as a sonic hedgehog (SHH) receptor and regulates SHH signalling at the primary cilium. SHH has an important role in embryonic development by establishing cell fate in the neural tube, somites, and limbs. In the absence of SHH, PTCH1 represses the function of the seven-pass transmembrane protein, Smoothened (SMO). SHH binding to PTCH1 inhibits this repression of SMO, which results in the activation of the GLI (Glioblastoma-associated oncogene) family transcription factors.

Heterozygous germline *PTCH1* mutations cause Gorlin syndrome (Gorlin-Goltz syndrome, nevoid basal cell carcinoma, or basal cell nevus syndrome), an autosomal dominant disorder that predisposes affected individuals to developmental defects and tumorigenesis. Dozens of pathogenic variants in the *PTCH1* gene include deletions or insertions, (multi)exon or large-scale deletions or rearrangements resulting in frameshifts, nonsense mutations leading to premature stops, missense, splice-site mutations, and deep intronic variants that alter splicing. A careful analysis revealed that high frequency of mutations is clustered into the intracellular and two extracellular loops Indeed, the *de novo* heterozygous variant, c.2834delGinsAGATGTTGTGGACCC, p.(Arg945GlnfsTer22) in our patient is located to one of the extracellular loops, leading to protein truncation, or nonsense-mediated mRNA decay. Either way, the attachment of SHH to the receptor PTCH1 and the suppression of SMO by PTCH1 might be impaired resulting in dysregulation of the SHH signalling pathway.

Experimental data on *PTCH1 (patched homolog 1)*

Shh is essential for ventral cell specification and various neural progenitor processes during prenatal and postnatal brain development. Consistent with the role of Shh in brain development, patched homolog 1 (Ptch1, PTCH1) the putative receptor for

Shh protein plays also an important role in neural development. Experimental studies showed expression of Ptch1 protein throughout the mouse embryo. It was bound to Shh, or other hedgehog family members and formed a complex with Smo. Mice heterozygous for the *Ptc* mutation were larger than normal, and a subset of them developed hind limb defects or cerebellar medulloblastoma. These studies suggest that the balance between Shh and Ptc activities appears critical for normal development and neural fate determination.

The role of *PTCH1* in oncogenesis

Experiments revealed that inactivation of the *Ptc* gene either in cerebellar granule neuron precursors or multipotent neural stem cells led to Shh pathway activation and medulloblastoma. As already mentioned patients with *PTCH1* mutations have high risk of medulloblastoma and/or basal cell carcinoma due to SMO activation and increased transcription of SHH-pathway target genes. Proliferation of cerebellar granule cell precursors that give rise to SHH-medulloblastoma is regulated by SHH signalling during the third trimester to the first few months of life in human. Interestingly, the lateral cerebellum is preferentially sensitive to high sonic hedgehog signalling and 59% of tumours with *PTCH1* mutation develop in the cerebellar hemispheres.

Coexistence of *FOXP1* and *PTCH1* mutations

Heterozygous disruptions in both *FOXP1* and *PTCH1* genes were found in our patient with extreme megalencephaly, partial callosal agenesis and profound intellectual disability. Both common and distinct features described in *FOXP1* or *PTCH1* mutations could be recognized. We suggest that the effects of multiple hits, disruptive disease-causing variants in two different genes simultaneously added and resulted in the serious composite clinical phenotype in our patient. *FOXP1* mutation might contribute to the severe intellectual disability, while the extreme megalencephaly in association with the partial callosal agenesis might rather be related to the *PTCH1* mutation.

Activating mutations in the PI3K (phosphatidylinositol-3-kinase)–AKT (*AK* mouse + *T*ransforming or *T*hymoma)-mTOR pathway and megalencephaly.

Mutations of upstream (*PIK3R2, PIK3CA, PTEN*), central (*AKT3, TSC1, TSC2, MTOR, DEPDC5*) and downstream (*CCND2*) genes within the PI3K-AKT-mTOR pathway are associated with brain overgrowth (megalencephaly), as well as cortical dysplasia (such as hemimegalencephaly, focal cortical dysplasia and polymicrogyria). Mutations in these genes lead to activation of the PI3K-AKT-mTOR pathway. The members of this pathway regulate a wide range of processes, including cell growth,

proliferation, survival, migration, metabolism, angiogenesis, apoptosis, tumorigenesis and brain development.

***AKT3* gene**

Mutations in *AKT3* are responsible for ***megalencephaly-polymicrogyria-polydactyly-hydrocephalus syndrome 2; MPPH2, OMIM 615937).*** *AKT3* consists of 24 exons and encodes a protein with 479 amino acids. Before 2017, *constitutional* mutations of *AKT3* in association with megalencephaly and polymicrogyria and *mosaic* mutations in hemimegalencephaly were reported in a few cases. Eventually a wide spectrum of developmental disorders found in 11 children reported previously and 14 newly diagnosed individuals with either constitutional or mosaic mutations in *AKT3* have been reviewed. Patients with constitutional mutations have been segregated into three groups, such as (i) megalencephaly-polymicrogyria, including our patient, (ii) megalencephaly-polymicrogyria with periventricular heterotopia, and finally (iii) megalencephaly with no or subtle cortical malformation and autismus spectrum disorder. Therefore, the phenotype associated with *AKT3* mutations proved to be wider than the megalencephaly-polymicrogyria-polydactyly-hydrocephalus syndrome 2 as suggested in the OMIM.

The constitutional *de novo* heterozygous missense variant, c.1393C>T; p.(Arg465Trp), found in our patient has been published only in 7 individuals so far. It is an activating mutation affecting the function of proteins that are involved in the regulation of diverse cellular functions, such as metabolism, translation, proliferation, survival, and angiogenesis.

Experimental data on *AKT3* gene

It was demonstrated that cells with the morphology of cytomegalic neurons in hemimegalencephaly due to mosaic *AKT3* mutation [c.49G>A; p.(Glu17Lys)] showed signs of activation of the PI3K-AKT-mTOR pathway. Further experiments on surgical brain tissue from hemimegalencephaly and focal cortical dysplasia also exhibited an activation of phosphorylation of the AKT protein. Kinase activity analysis also clearly showed that all mutations causing megalencephaly, including the constitutional variant, found in our patient caused increased activity compared to wild type, confirming that these mutations are activating the PI3K-AKT-mTOR pathway.

The PI3K–AKT-mTOR pathway in oncogenesis

The PI3K-AKT-mTOR pathway is one of the most commonly dysregulated pathways in all of cancer. Mutations of genes encoding the tyrosine kinase receptors, or the members of the PI3K pathway represent very common variants in oncology. Forty-

sixty percent of primary melanomas were found to have increased total or phosphorylated AKT3 protein compared to normal melanocytes. Considering the findings regarding the relationship between PI3K-AKT-mTOR pathway activation and oncogenesis, careful monitoring of Patient 5 for cancer is warranted.

CONCLUSIONS

This study provides phenotypic and genotypic characterization of three patients with microcephaly and two patients with megalencephaly. Next generation sequencing revealed novel homozygous pathogenic variants in the *ASPM* and *WDR62* genes causing autosomal recessive primary microcephaly (MCPH). These mutations might lead to abnormal function of the centrosomes, which organize the separation of chromosome copies during cell division. Coexistence of heterozygous *de novo* variants in *FOXP1* and *PTCH1* genes was found in association with extreme megalencephaly and profound intellectual disability, as a composite phenotype, sharing some features of Gorlin syndrome. Activating heterozygous *de novo* mutations in *AKT3,* a gene encoding a protein in the PI3K-AKT-mTOR pathway resulted in polymicrogyria and megalencephaly. There is evidence that abnormalities in the SHH-PTCH1 and PI3K-AKT-mTOR pathway have significant role in oncogenesis, therefore our patients with brain overgrowth require regular medical follow-up. Autosomal recessive primary microcephaly and megalencephaly are rare diseases and our study is in line with the international efforts of discovering the molecular background of these disorders.

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