Supplementary Information

De novo germline and postzygotic mutations in *AKT3*, *PIK3R2* and *PIK3CA* cause a spectrum of related megalencephaly syndromes

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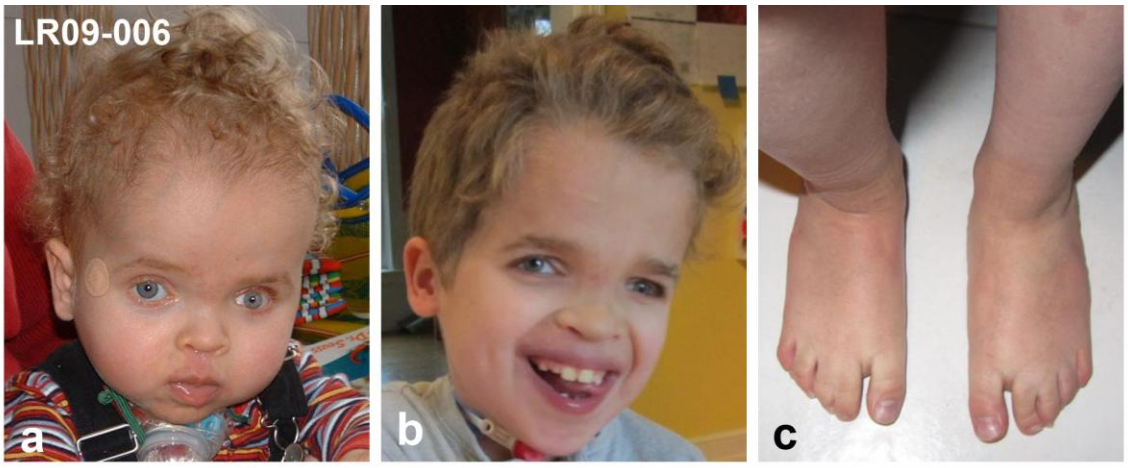
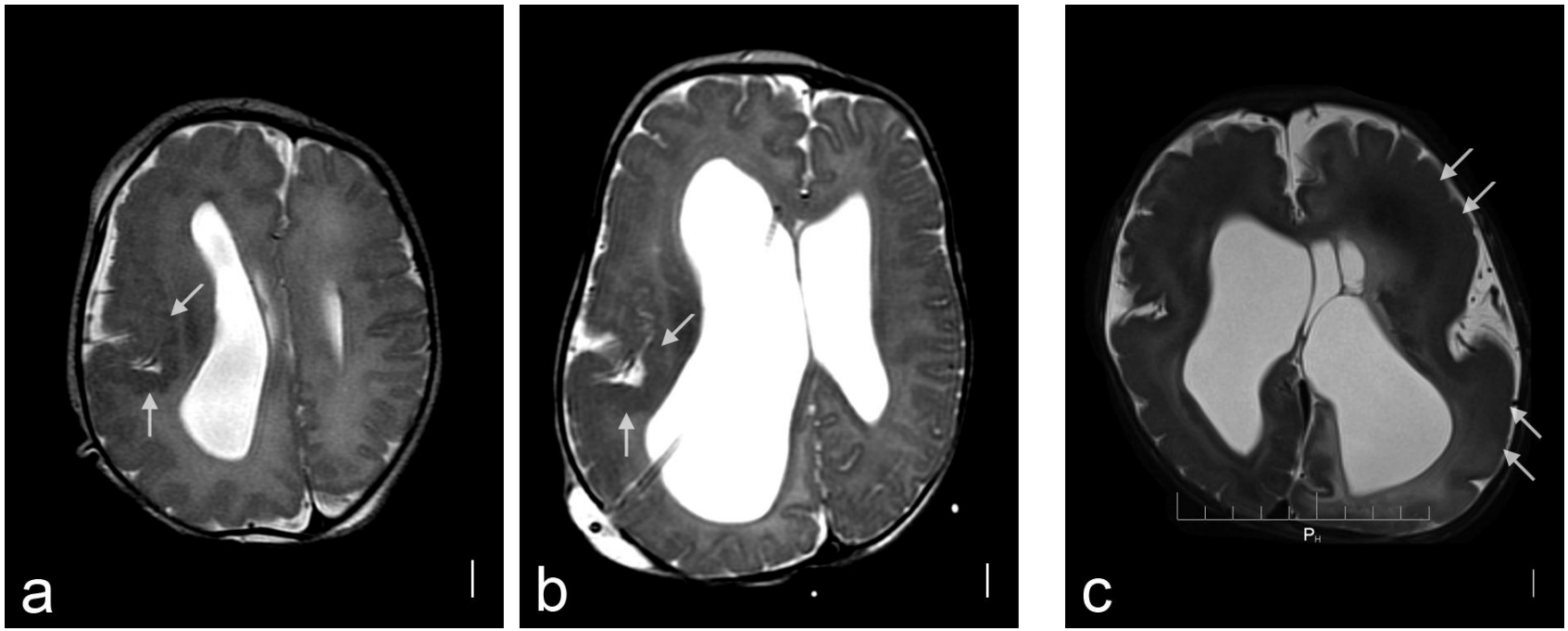
SUPPLEMENTARY FIGURES

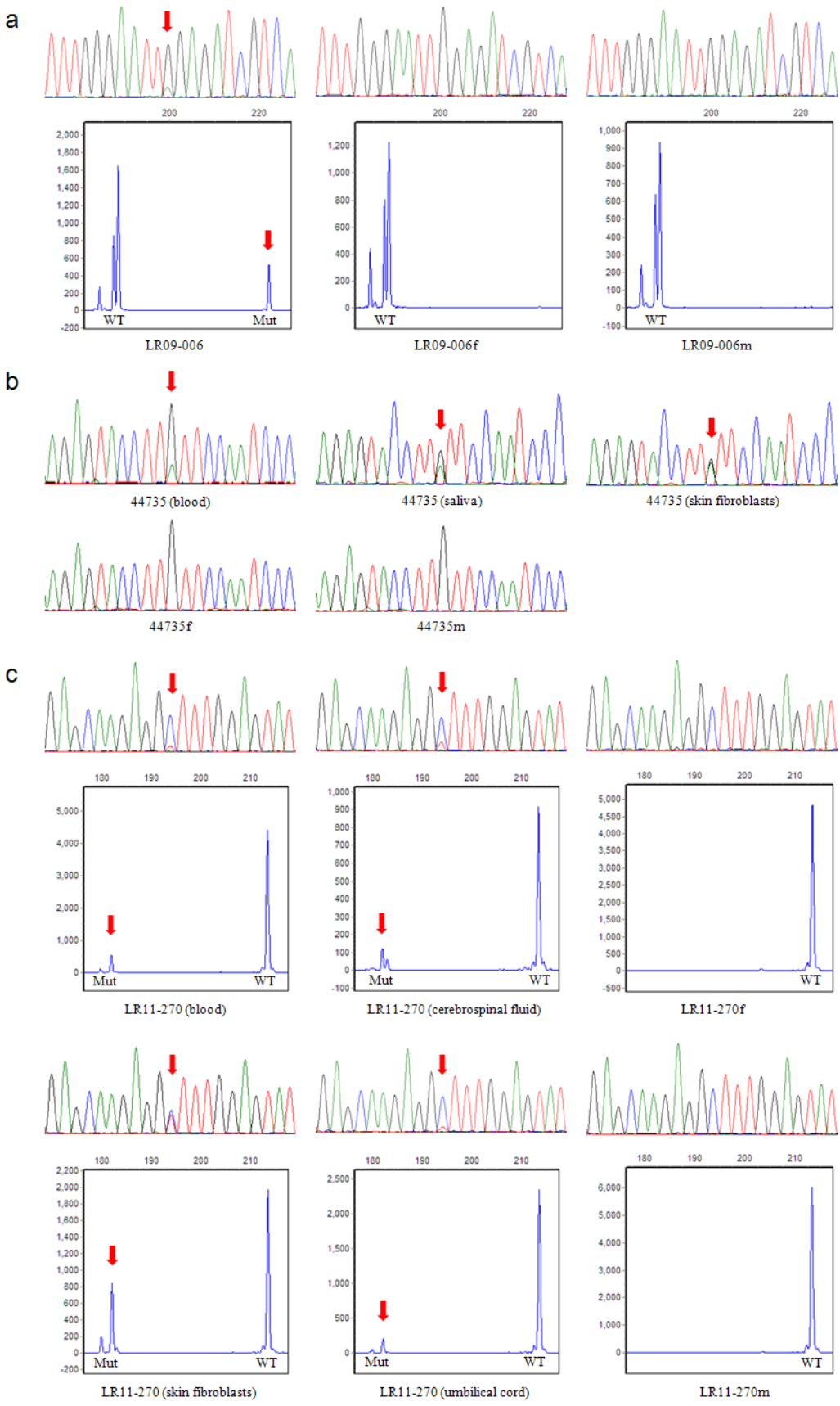


Supplementary Figure 1. Photos of the face and feet of patient LR09-006 at 15 months (**a**), 7.5 years (**b**), and 8.5 years (**c**). Note the prominent wide forehead, and midline facial nevus flammeus at 15 months that later disappeared at 7.5 years. The patient also has bilateral 2-3 toe syndactyly with sandal-gap toes (**c**). We obtained written consent to publish photographs of the patient.

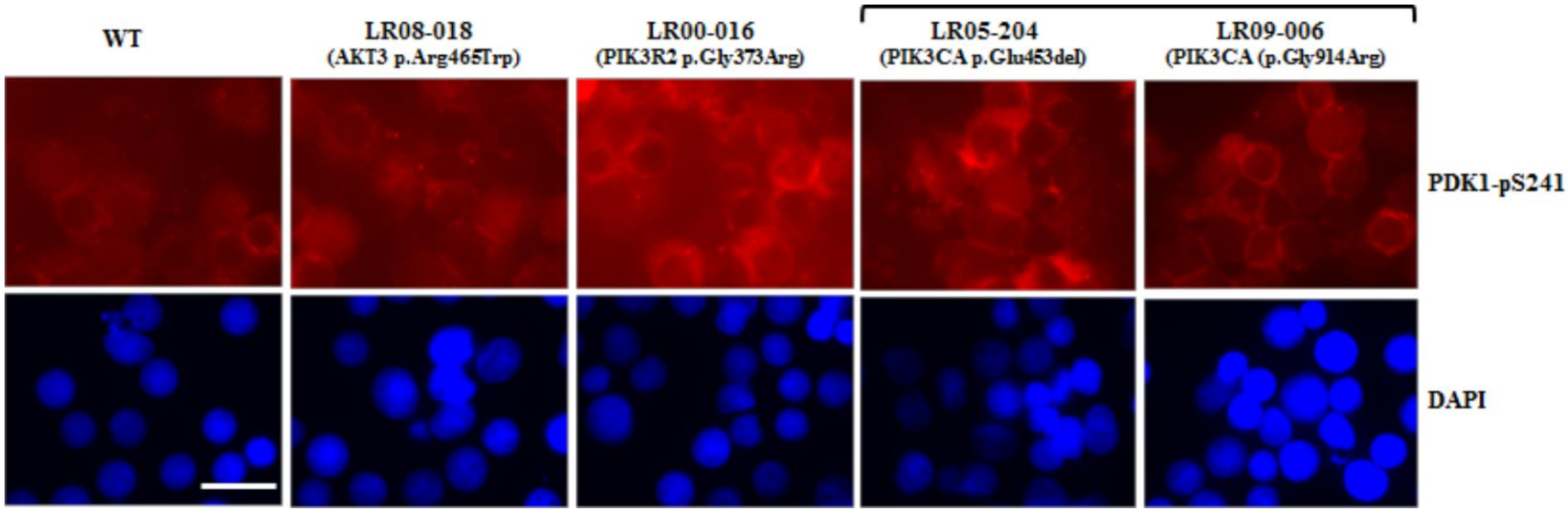


Supplementary Figure 2. Hemimegalencephaly (HMEG) and "bilateral" HMEG in patients with mutations of *PIK3CA*. Brain MRI in patient LR11-069 (*PIK3CA*, p.Thr1025Ala) at 3 days **(a)** and 1 month **(b)** of age show HMEG with marked enlargement and extensive cortical malformation of the right hemisphere. The cortical malformation has the irregular or pebbled appearance typical of polymicrogyria, and appears most severe in the right posterior frontal and perisylvian regions (arrows in **a** and **b**). Other images (not shown) demonstrate a subtle cortical malformation in the left hemisphere as well, which is not unusual in HMEG. This appearance supports a recent report of duplication or intragenic mutation of *AKT3* in three patients with isolated HMEG[1]. **(c)** Brain MRI at 12 months in patient LR12-033 (*PIK3CA*, p.Glu545Lys) shows a severe cortical malformation resembling the most severe form of HMEG on both sides of the brain. The key features include abnormally wide gyri (pachgyria) and moderately thick cortex with a

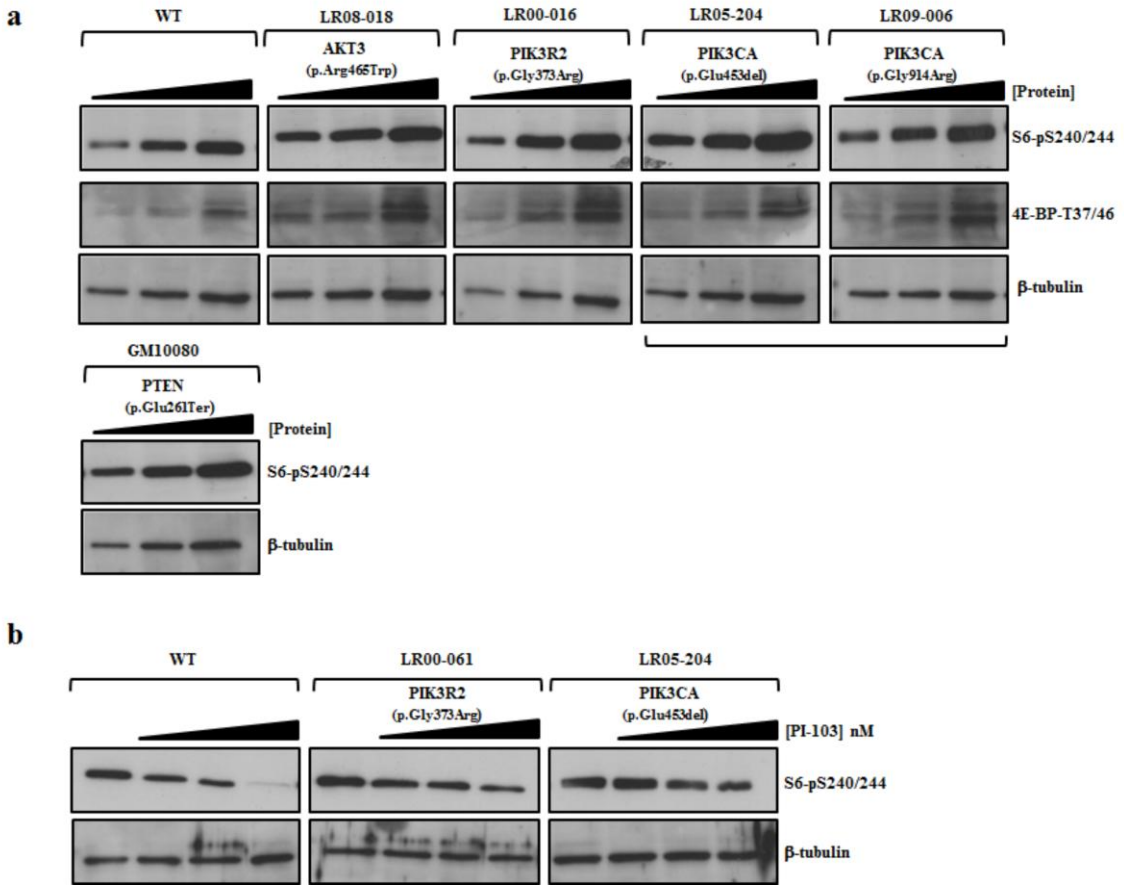
smooth rather than pebbled or polymicrogyric surface that appears more severe on the left (arrows) than right side. The white matter is poorly myelinated with unusual bands of myelination in the periventricular regions, and the lateral ventricles are moderately enlarged with a possible cyst in the posterior left lateral ventricle. These changes are more severe than other patients with MCAP or MPPH. Scale bars correspond to 1 cm.


a
WT
Mut
LR09-006
WT
LR09-006f
WT
LR09-006m
b
44735 (blood)
44735 (saliva)
44735 (skin fibroblasts)
44735f
44735m
c
Mut
WT
LR11-270 (blood)
Mut
WT
LR11-270 (cerebrospinal fluid)
WT
LR11-270f
Mut
WT
LR11-270 (skin fibroblasts)
Mut
WT
LR11-270 (umbilical cord)
WT
LR11-270m

Supplementary Figure 3. Examples of *PIK3CA* mutations. Sanger sequence traces and restriction-enzyme assay electropherograms of three mosaic mutations: (**a**) p.Gly914Arg in subject LR09-006 (LR09-006f: father, LR09-006m: mother); (**b**) p.Cys378Tyr in subject 44735 (44735f: father, 44735m: mother); and (**c**) p.Ala1035Val in subject LR11-270 (LR11-270f: father, LR11-270m: mother). Red arrows indicate the position of the mutant allele. Mutations p.Cys378Tyr and p.Ala1035Val show varying levels of the mutant allele depending on the tissue tested. Estimates of mutant allele levels are shown in **Supplementary Table 4**.



Supplementary Figure 4. Indirect immunofluorescence (IF) staining of active PDK1 in exponentially growing lymphoblastoid cell lines using an anti-PDK1-p Ser241 antibody (see **Online Methods**). Images were captured on the Zeiss AxioPlan platform using the same exposure times for each sample. Activated PDK1 is enriched at the cell membrane by binding to PIP3. Mutant lymphoblastoid cell lines derived from individuals LR00-016a1 (*PIK3R2* p.Gly373Arg), LR05-204 (*PIK3CA* p.Glu453del) and LR09-006 (*PIK3CA* p.Gly914Arg) all appear to exhibit increased membrane-localized endogenous PDK1-pSer241 compared to wild-type (WT) and *AKT3* mutant (p.Arg465Trp) cells. Scale bar corresponds to 10μm.



Supplementary Figure 5. Western blot analysis of phosphorylated S6 and 4E-BP1 in mutant and control lymphoblastoid cell lines. **(a)** Endogenous levels of phosphorylated S6 and 4E-BP1 in differing amounts of whole cell extracts (5, 10, and 25 μg) using phospho-specific antisera against S6-pSer240/244 and 4E-BP-pThr37/47 (see **Online Methods**). All mutant lymphoblastoid cell lines exhibited elevated levels of phosphorylated S6 and 4E-BP1 compared to wild-type (WT). Elevated S6-pSer240/244 was also evident in GM10080, a lymphoblastoid cell line from a patient with Cowden disease which served as positive control. **(b)** S6 phosphorylation was reduced to a greater extent in control cells compared to those with *PIK3R2* (LR00-016a1 p.Gly373Arg) or *PIK3CA* (LR05-204 p.Glu453del) mutations upon exposure to differing amounts of PI-103 (0, 50, 100, 200nM) for 2 hours, thus further reflecting elevated PI3K-mTOR signaling in mutant cell lines.

SUPPLEMENTARY TABLES

Supplementary Table 1. Summary of the exome sequencing results

Sample ID	Target size[a]	Mappable sequence[b]	Depth of coverage		Percent target ≥ 4x		Percent target ≥ 10x		Total variants[c]	Rare Variants[d]
			Mean	Median	Per sample	Cumulative	Per sample	Cumulative		
LR08-018	50	7.4	68	57	92.6	90.3	88	83.4	36,618	254
LR08-018f	50	8.5	77	74	92.3		87.5		-	-
LR08-018m	50	7.6	67	51	92.4		86.4		-	-
LR09-006	36.7	5.5	74	65	94.8	92.7	91.1	87.6	34,506 (39,071)	251 (291)
LR09-006f	36.7	9.9	130	94	94.2		90		-	-
LR09-006m	36.7	5.9	79	65	93.7		89.1		-	-
LR00-016a1	36.7	5.1	75	61	93.1	-	88.2	-	32,751	247

[a]In megabases. [b]In gigabases. [c]Variants that passed on the GATK hard-filtering parameters used for variant calling. For LR09-006, numbers in parentheses also include variants that failed on the hard-filtering parameters. [d]Protein-altering and splice-site variants absent from 112 other exomes, dbSNP (build 132), and the 1000 Genomes Project data[2]. Coverage metrics were calculated using the GATK[3] Depth of Coverage tool by considering only reads with mapping quality ≥ 20 and bases with base quality ≥ 20.

Supplementary Table 2. Candidate *de novo* events for subjects LR08-018 and LR09-006

Trio	Gene	Coordinates (hg19)	Type of change	Amino acid change	Proband reads[a]	Father reads[a]	Mother reads[a]	Mutant allele (%)	Filter[b]	Sanger Validation
LR08-018	*AKT3*	chr1:243668598 G>A	missense	p.Arg465Trp	23/54	0/63	0/47	43	PASS	*De novo*
	PCDHGA3	chr5:140723988 (3-bp del)	in-frame	-	67/147	0/195	0/111	46	PASS	Not tested
LR09-006	*BSN*	chr3:49680130 G>A	missense	p.Ala355Thr	6/14	0/14	1/5	43	PASS	Not tested
	PIK3CA	chr3:178947865 G>A	missense	p.Gly914Arg	20/177	0/363	0/204	11	Filtered	*De novo*
	MUC4	chr3:195506712 (48-bp del)	in-frame	-	7/46	0/16	0/43	15	Filtered	Not tested
	MUC4	chr3:195509346 (48-bp del)	in-frame	-	5/17	0/19	0/18	29	PASS	Not tested
	MUC4	chr3:195511042 G>A	missense	p.Thr2470Ile	4/20	0/40	1/25	20	Filtered	Not tested
	MUC4	chr3:195511046 C>T	missense	p.Asp2469Asn	4/23	0/36	0/24	17	Filtered	Not tested
	MUC4	chr3:195511060 G>C	missense	p.Pro2464Arg	3/19	0/33	0/21	16	Filtered	Not tested
	MUC4	chr3:195512242 G>A	missense	p.Pro2070Leu	3/9	1/30	0/6	33	Filtered	Not tested
	BDP1	chr5:70757754 G>A	splice-site	-	15/32	1/65	0/27	47	PASS	*De novo*
	MICA	chr6:31380161 (11-bp ins)	frameshift	-	17/165	0/166	0/42	10	Filtered	Not tested
	PRDM10	chr11:129780428 (6-bp del)	in-frame	-	6/19	0/21	0/13	32	PASS	Not tested
	KRT82	chr12:52788781 C>G	missense	Ser507Thr	2/7	0/4	1/6	29	Filtered	Not tested
	RGS11	chr16:320630 C>A	missense	p.Trp345Cys	2/9	0/14	0/9	22	Filtered	Not tested
	LMF1	chr16:921179 G>A	missense	p.Arg354Trp	4/17	1/10	0/6	24	Filtered	Not tested

Abbreviations: del: deletion; ins: insertion. [a]The numerator is the number of reads supporting the variant; the denominator indicates the total number of reads at the variant site. [b]Indicates if a variant passes ("PASS") or fails ("filtered") on the hard-filtering parameters used for variant calling (**Online Methods**).

Supplementary Table 3. Candidate genes for family LR00-016 assuming autosomal recessive inheritance

Exome sequencing data				Genome-wide SNP data (Illumina 610K)					
Gene symbol	**Variant genotype**	**Genomic DNA change (hg19)**	**Amino acid change**	**Shared alleles[a]**	**Number of SNPs[b]**	**Proximal recombinant**	**Distal recombinant**	**Shared interval (hg19)**	**Size of interval[c]**
UNC80	heterozygous heterozygous	chr2:210678385 G>T chr2:210678386 C>T	p.Gln340His p.Pro341Ser	yes	11	rs2370833	rs7608462	chr2:210669245-210748646	79,401
ZNF717	homozygous	chr3:75790448 T>C	p.Thr86Ala	no					
MUC4	heterozygous heterozygous	chr3:195507461 G>A chr3:195508127 G>C	p.Pro3536Ser p.Pro3314Ala	yes	12	rs2688492	rs2641772	chr3:195496129-195531841	35,712
RREB1	heterozygous heterozygous	chr6:7182247 G>A chr6:7229275 C>T	p.Gly35Arg p.Arg315Cys	no					

Abbreviations: SNP: single-nucleotide polymorphism. [a]Presence of at least two consecutive SNPs with shared genotypes across all three affected siblings. [b]Number of SNPs flanking the candidate mutations with shared genotypes across all three affected siblings. [c]In base-pair.

Supplementary Table 4. Summary of the genomic DNA samples tested, methods used, estimated levels of mosaic mutations in *PIK3CA*, and mutant allele frequency in 174 control exomes

Sample Information				Methods and estimated levels of mutant allele (%)				Control exomes (n = 174)			
Sample ID	**Mutation**	**Discovery method**	**Source of DNA**	**Sanger sequencing**	**Fluorescent assay**	**Exome sequencing**	**Deep sequencing**	**Mean (median) coverage[a]**	**Total alt allele freq ($x10^{-4}$)[b]**	**Controls with alt allele[c]**	**Max alt/total reads[d]**
LR06-342	p.Glu81Lys	Deep seq	Saliva	Undetected			3-8	72 (72)	0.8	1	1/80
			LCL	Undetected			1				
LR06-220	p.Arg88Gln	Sanger	Blood	50			43	70 (71)	1.6	2	1/60
			Saliva	50							
LR11-068	p.Arg88Gln	Deep seq	Blood	Undetected			1-2	70 (71)	1.6	2	1/60
			Saliva	Undetected			4				
07-0388	p.Gly364Arg	Exome	Blood	17		15		469 (217)	0.6	5	1/1064
			Saliva	23							
LR05-139	p.Glu365Lys	Sanger	Blood	15				481 (228)	0.2	2	1/344
			Saliva	38							
44735	p.Cys378Tyr	Exome	Blood	19		27	30	419 (160)	1.2	8[e]	1/88
			Saliva	35							
			Skin fibr	46							
LR11-153	p.Glu453del	Sanger	Blood	50	49			-	-	-	-
			Saliva	50	50						
LR05-204	p.Glu453del	Sanger	Blood	10	11			-	-	-	-
LR12-033	p.Glu545Lys	Sanger	Blood	Undetected				64 (62)	1.8	2	1/58
			Buccal swab	27							
162-001P	p.Glu726Lys	Exome	Blood	10	7	9		93 (93)	0.6	1	1/89
			Saliva	28	26						
LR08-261	p.Glu726Lys	Sanger	Blood	12	20		12	93 (93)	0.6	1	1/89

			Buccal swab	28	48		41				
LR06-333	p.Glu726Lys	Sanger	LCL	14	23		14	93 (93)	0.6	1	1/89
			Saliva	16	20		15				
LR09-006	p.Gly914Arg	Exome	Blood	13	17	11	16	144 (139)	1.2	3	1/153
LR11-070	p.Gly914Arg	Sanger	Blood	10	8			144 (139)	1.2	3	1/153
			LCL	16	17		15				
			Saliva	20	15		17				
LR06-341	p.Gly914Arg	Sanger	Blood	15	15		15	144 (139)	1.2	3	1/153
			LCL	38	33						
			Saliva	21	17		19				
11-0117	p.Gly914Arg	Exome	Blood	7	9	5		144 (139)	1.2	3	1/153
LR11-212	p.Tyr1021Cys	Sanger	Saliva	18				82 (85)	0.7	1	1/123
LR11-069	p.Thr1025Ala	Sanger	LCL	24			24	80 (86)	0.7	1	1/97
			Saliva	10			12				
LR11-270	p.Ala1035Val	Sanger	Blood	14	13			86 (92)	2.0	3	1/35
			Skin fibr	44	33						
			UC	14	10						
			CSF	21	17						
115422	p.Ala1035Val	Exome	Blood	Undetected	Undetected	2		86 (92)	2.0	3	1/35
			Saliva	17	16						
			Buccal swab	18	8						
			Skin fibr	Undetected	Undetected						
86708	p.Met1043Ile	Exome	Blood	Undetected	Undetected	3		89 (96)	2.6	4	1/59
			Skin fibr	35	23						
121939	p. His1047Tyr	Exome	Blood	8	4	6		88 (93)	2.6	4	1/40
			Buccal swab	15	9						
LR11-285	p. His1047Tyr	Sanger	Saliva	22	17			88 (93)	2.6	4	1/40

LR11-230	p.Gly1049Ser	Sanger	Saliva	40	84 (90)	0.7	1	1/77

Abbreviations: Deep seq: ultra-deep sequencing; CSF: cerebrospinal fluid; LCL: lymphoblastoid cell line; Skin fibr: skin fibroblasts; UC: umbilical cord. **Controls exomes**: [a]average and median coverage at the mutation site using a base quality threshold of 20. [b]Frequency of the mutant allele across all 174 control exomes. [c]Number of control individuals with at least one read with the mutant allele. [d]Control individual with the highest level of mutant allele (the numerator indicates the number of reads supporting the variant; the denominator is the total number of reads at the variant site). [e]One control had 2 reads supporting the mutant allele (2 out of 1,428 total reads).

Supplementary Table 5. Overview of *PIK3CA* variant sites supported by at least 1, 2, 3 or 4 reads in exome sequencing data from 6 MCAP patients and 174 unrelated control individuals

Cohort	**Total samples**	**≥ 1 variant reads**	**≥ 2 variant reads**	**≥ 3 variant reads**	**≥ 4 variant reads**
		Samples (sites)	**Samples (sites)**	**Samples (sites)**	**Samples (sites)**
MCAP patients	6	6 (85)	6 (26)	6 (8)	5 (5)
Controls	174	174 (4349)	108 (276)	4 (4)	0 (0)
Fisher's exact test			$P = 0.09$	$P = 4.8 \times 10^{-9}$	$P = 4.0 \times 10^{-9}$

Candidate variant sites were identified using a base quality threshold of 20 and a variant read frequency ≥ 1%. The "Samples (sites)" columns indicate the number of individuals with at least one variant site, and the total number of variant sites (in parentheses). Fisher exact probability tests (two-tailed) are based on the number of patients and control individuals with/without variant sites.

Supplementary Table 6. *PIK3CA* variant sites supported by at least 3 reads in exome sequencing data from 6 MCAP patients and 174 control individuals

Sample ID	Mutation coordinates (hg19)	Type of change	Amino acid change	Alt/total reads[a]	Percent mutant allele	Validation[b]
1. MCAP patients (n=6)						
07-0388	chr3:178922321 G>A	missense	p.Gly364Arg	33/220	15	Confirmed
162_001P	chr3:178938934 G>A	missense	p.Glu726Lys	7/78	9	Confirmed
162_001P	chr3:178943798 A>T	missense	p.Asn822Ile	3/78	4	Undetected
162_001P	chr3:178952042 C>A	missense	p.Gln1033Lys	3/176	2	Undetected
11-0117	chr3:178947865 G>A	missense	p.Gly914Arg	17/320	5	Confirmed
115422	chr3:178952049 C>T	missense	p.Ala1035Val	3/185	2	Confirmed
86708	chr3:178952074 G>T	missense	p.Met1043Ile	6/201	3	Confirmed
121939	chr3:178952084 C>T	missense	p.His1047Tyr	10/170	6	Confirmed
2. Control individuals (n=174)						
11291m	chr3:178927469 G>T	missense	p.Gly411Val	3/133	2	Not tested
13530m	chr3:178927471 C>T	nonsense	p.Arg412Ter	3/194	2	Not tested
11198f	chr3:178936985 C>T	missense	p.His556Tyr	3/198	2	Not tested
11653f	chr3:178938833 G>T	missense	p.Cys692Phe	3/79	4	Not tested

[a]The numerator refers to the number of reads supporting the variant; the denominator indicates the total number of reads at the variant site (only bases with base quality ≥ 20 were considered). [b]See **Supplementary Table 4** for details on the validation experiments.

Supplementary Table 7. Overview of the deep sequencing experiment

Run[a]	Targeted region		Experimental design			Summary of results		
	Mutation	Target Size (bp)[b]	Unexplained cases	Mutation carriers[d]	Negative controls	Mean coverage (median)[e]	New mutations	Confirmed mutations
1	p.Arg88Gln	66	15	1	4	148,135 (104,537)	2/15	1/1
1	p.Cys378Tyr	70	15	1	4	236,759 (201,005)	0/15	1/1
1	p.Glu726Lys	66	14[c]	2 (4)	5	102,815 (98,195)	0/14	2/2
1	p.Gly914Arg	59	15	3 (5)	6	288,054 (255,417)	0/15	3/3
1	p.Thr1025Ala	68	15	1 (2)	2	44,951 (30,695)	0/15	1/1
2	p.Glu81Lys p.Arg88Gln	66	0	2 (4)	2	135,054 (112,497)	-	2/2

[a]Run 1: initial experiment; run 2: validation of newly identified mutations. [b]Excluding primer regions. [c]Originally 15 individuals; one sample failed. [d]Numbers in parentheses indicate the number of tissues tested. [e]Only bases with base quality ≥ 20 were considered.

Supplementary Table 8. Summary of the phenotypic features of *AKT3*, *PIK3R2*, and *PIK3CA* mutation carriers

Basic data					Clinical features						Neuroimaging features		
Group	**Patient ID**	**Gene**	**Sex**	**Age last assessed**	**Last OFC-SD (age)**	**OG /ASYM**	**Vascular**	**SYN**	**POLY**	**CONN**	**HYD/ VMEG**	**CBTE**	**PMG**
Overlap	LR08-018	*AKT3*	M	11m	+5.5 (7.5m)	nd/–	+	–	–	+	+	+	+
MPPH	LR11-354	*AKT3*	F	2y5m	+6 (2y5m)	–/–	–	–	–	+	+	–	+
MPPH	LR00-016a1	*PIK3R2*	M	16y	+5 (13y)	–	–	–	–	–	+	+	+
MPPH	LR00-016a2	*PIK3R2*	F	14y	+5 (12y)	–	–	–	–	–	+	+	+
MPPH	LR00-016a3	*PIK3R2*	F	12y	+4 (10y)	–	–	–	–	–	+	+	+
MPPH	LR01-164	*PIK3R2*	M	8m	+4 (8m)	nd	–	–	–	–	+	–	+
MPPH	LR04-069	*PIK3R2*	M	9y	+8 (9y)	+/–	–	–	+	–	+	–	+
MPPH	LR04-032	*PIK3R2*	F	4y	+5-6 (4y)	+/–	–	–	–	–	+	–	+
MPPH	LR04-373	*PIK3R2*	F	5y	+6 (3y6m)	nd	–	–	–	–	+	–	+
MPPH	LR08-263	*PIK3R2*	M	9y	+6 (9y)	–/–	–	–	–	–	+	+	+
MPPH	LR08-422	*PIK3R2*	M	8.5m	+5 (8.5m)	–/–	–	–	–	–	+	+	+
MPPH	LR04-181	*PIK3R2*	F	7y	>2 (7y)	–/–	–	–	–	–	+	+	+
MPPH	LR11-021	*PIK3R2*	F	4y	+4 (4y)	–/–	–	–	–	–	+	+	+
MPPH	LR11-204	*PIK3R2*	M	8m	+4 (8m)	–/–	–	–	+	–	+	–	+
MPPH	LR11-353	*PIK3R2*	F	4y6m	+6 (4y)	–/–	–	–	–	–	+	+	+
MCAP	LR06-342	*PIK3CA*	M	2y	+2 (birth)	+/+	+	+	+	+	+	+	–
MCAP	LR06-220	*PIK3CA*	F	6y	+4 (6y)	–/–	+	–	–	–	–	–	+
MCAP	LR11-068	*PIK3CA*	M	19m	+4 (19m)	+/+	+	–	–	+	nd	nd	+
MCAP	07-0388	*PIK3CA*	M	7y6m	+4-5 (7y6m)	+/+	+	+	–	+	+	+	–
MCAP	LR05-139	*PIK3CA*	M	4y6m	+8 (9m)	+/–	+	+	–	+	+	+	+
MCAP	44735	*PIK3CA*	F	9y	+8 (9y)	+/+	+	+	–	+	+	+	+
MCAP	LR11-153	*PIK3CA*	M	16m	+5 (12m)	+/+	+	+	+	+	+	+	+
MPPH	LR05-204	*PIK3CA*	M	4y	+9-10 (4y)	+/–	–	–	–	+	+	+	+

Overlap	LR12-033	*PIK3CA*	F	16m	+4 (16m)	+/+	–	–	+	+	+	–	+
MCAP	162-001P	*PIK3CA*	M	6y	+5 (6y)	+/–	+	–	–	+	+	+	+
MCAP	LR08-261	*PIK3CA*	F	1y11m	+7-8 (2m)	+/–	+	–	–	+	+	+	+
MCAP	LR06-333	*PIK3CA*	M	5y6m	nd	–/–	+	+	–	+	+	+	+
MCAP	LR09-006	*PIK3CA*	M	5y6m	+9-10 (5y)	+/+	+	+	+	+	+	+	+
MCAP	LR11-070	*PIK3CA*	F	3y	+4 (3y)	+/–	+	–	–	+	+	+	nd
MCAP	LR06-341	*PIK3CA*	M	3y7m	nd	+/+	+	–	–	+	+	+	+
MCAP	11-0117	*PIK3CA*	F	1y5m	+5.5 (1y5m)	+/+	+	–	–	+	+	+	–
MCAP	LR11-212	*PIK3CA*	M	28m	+2 (2y)	–/+	+	–	+	+	+	+	–
MCAP	LR11-069	*PIK3CA*	F	6m	+5-6 (6m)	+/+	+	–	–	+	+	–	+
MCAP	LR11-270	*PIK3CA*	M	10m	+4 (10m)	+/–	+	+	+	nd	+	+	+
MCAP	115422	*PIK3CA*	F	3y6m	+5-6 (3y6m)	+/+	+	–	–	–	+	+	+
MCAP	86708	*PIK3CA*	M	15y	+5 (15y)	+/+	+	–	–	–	–	+	+
MCAP	121939	*PIK3CA*	M	3y8m	+5 (3y8m)	+/+	+	+	–	+	+	+	+
MCAP	LR11-285	*PIK3CA*	M	6m	+2 (2m)	+/–	+	–	–	–	+	+	+
MCAP	LR11-230	*PIK3CA*	M	12m	+10 (1y6m)	+/–	+	+	–	+	+	+	+

Abbreviations: ASYM: asymmetry; CBTE: cerebellar tonsillar ectopia; CONN: connective tissue dysplasia (including skin laxity, joint hypermobility, thick doughy subcutaneous tissue); F: female; HYD: hydrocephalus; M: male; nd: no data; OFC: occipito-frontal circumference; OG: overgrowth; PMG: Polymicrogyria; POLY: polydactyly; SD: standard deviations; SYN: syndactyly; Vascular: vascular malformations; VMEG: ventriculomegaly.

Supplementary Table 9. Summary of explained and unexplained megalencephaly subjects

	AKT3	*PIK3R2*	*PIK3CA*	Unexplained	Total
MPPH	1	11	1	6	19
Overlap	1		1		2
MCAP			22	7	29
Total	2	11	24	13	50

Supplementary Table 10. Reports of cancer in MCAP and MPPH

Subject	Sex	Syndrome	Cancer	Age	Reference
1. Malignant tumors					
Patient 2	F	MCAP	Wilms tumor	10mo	Lapunzina *et al.*[4]
Patient 3	M	MCAP	Wilms tumor	4y	Wright *et al.*[5]
Not stated	U	MCAP	Leukemia	18y	Moore *et al.*[6]
Patient 2	M	MPPH	Medulloblastoma	2y	Osterling *et al.*[7]
2. Notable benign tumors					
Not stated	U	MCAP	Meningioma	21mo	Moore *et al.*[6]
Patient 14	M	MCAP	Meningioma	5y	Conway *et al.*[8]

Abbreviations: F, female: M, male; mo, months; U, unknown sex; y, years.

Supplementary Table 11. Oligonucleotide sequences for the restriction-enzyme and genotyping assays

Mutation	Oligonucleotides (5' → 3')	Restriction enzyme	Digested allele	Fragment size (bp)
p.Glu453del	FAM_TCTGGAAAAATGGCTTTGAA	-	-	WT: 238
	gtttcttCCACACTGCTGAACCAGTCA			Mut: 235
p.Glu726Lys	FAM-TTTGAAGCACCTGAATAGGC	BtsCI	WT	WT: 85
	gtttcttTGGGCTTCTAAACAACTCTGC			Mut: 168
p.Gly914Arg	gtttcttTACTGTGTAGCTACCTTCATTTTGGcAAT	MfeI	WT	WT: 190
	FAM-TTGTGATCCAAAAAGTGTCCA			Mut: 223
p.Ala1035Val	gtttcttGCCTTAGATAAAACTGAGCAAGAcG	HpyCH4IV	Mut	WT: 212
	FAM-CGGTCTTTGCCTGCTGAGAGT			Mut: 182
p.Met1043Ile	FAM-ATGATGCTTGGCTCTGGAAT	AseI	Mut	WT: 181
	gtttcttTGTGTGGAAGATCCAATCCAT			Mut: 119
p. His1047Tyr	FAM-ATGATGCTTGGCTCTGGAAT	NsiI	Mut	WT: 181
	gtttcttTGTGTGGAAGATCCAATCCAT			Mut: 129

Abbreviations: FAM: carboxyfluorescein; Mut: mutant allele; WT: wild-type allele. Unlabeled primers contain a GTTTCTT sequence on the 5' end (in lower case). The forward primers for mutations p.Gly914Arg and p.Ala1035Val were modified (indicated in lower case) to create a restriction site in the presence of the wild-type or the mutant allele, respectively.

Primers used to amplify the segmental duplication: forward primer, 5'-AATATCTCATGCTTGCTTTGGTTC-3'; reverse primer, 5'-TTACTAGAGGTGAGGAATGAGCTTC-3' (see **Online Methods** for details).

Supplementary Table 12. Oligonucleotide sequences for the targeted deep sequencing assay

Oligonucleotide name	**Sequence (5' → 3')**		
General format of primers[a]	Forward: AATGATACGGCGACCACCGAGATCTACACATACGAGATCCGTAATCGGGAAGCTGAAGyyyyyyyyyy		
	Reverse: CAAGCAGAAGACGGCATACGAGATxxxxxxxxACACGCACGATCCGACGGTAGTGTzzzzzzzzzz		
Mutation site	**Locus specific primers**	**Coordinates of target region (hg19)[b]**	**Amplicon size (target region)[c]**
p.Arg88Gln	Forward: TGTTACTCAAGAAGCAGAAAGG	chr3:178916851-178916916	222 (66)
	Reverse: CGGTTGCCTACTGGTTCAAT		
p.Cys378Tyr	Forward: ACCATGGAGGAGAACCCTTA	chr3:178922333-178922402	228 (70)
	Reverse: GCTAAACACTAATATAACCTTTGG		
p.Glu726Lys	Forward: AGGCAATGGAAAAGCTCATT	chr3:178938898-178938963	223 (66)
	Reverse: TGCAGTGAAAAGAGTCTCAAACA		
p.Gly914Arg	Forward: TTTACACGTTCATGTGCTGGA	chr3:178947835-178947893	217 (59)
	Reverse: CCATTACTTGTCCATCGTCTTTC		
p.Thr1025Ala	Forward: GATGCTTGGCTCTGGAATG	chr3:178951976-178952043	224 (68)
	Reverse: TCATGAAATACTCCAAAGCCTCT		

[a]Oligonucleotides contain Illumina adapter sequences, 8-mer barcodes (indicated by "x"), and locus specific primers (indicated by "y" and "z" for forward and reverse primers, respectively). [b]Excluding primer regions. [c]In base pair; amplicon size include locus specific primers, Illumina adapters and barcodes; numbers in parenthesis indicate the size of the target region (excluding locus specific primers and Illumina adapters).

SUPPLEMENTARY NOTE

FORGE Canada Consortium Steering Committee

Kym Boycott (leader; University of Ottawa), Jan Friedman (co-lead; University of British Columbia), Jacques Michaud (co-lead; Université de Montréal), Francois Bernier (University of Calgary), Michael Brudno (University of Toronto), Bridget Fernandez (Memorial University), Bartha Knoppers (McGill University), Mark Samuels (Université de Montréal), Steve Scherer (University of Toronto).

Ascertainment of study subjects

Written, informed consent was obtained from all participants, and the study was approved by the institutional review boards at participating institutions: the University of Chicago, Seattle Children's Hospital, Cedars-Sinai Medical Centre in Los Angeles, and Children's Hospital of Eastern Ontario Research Institute in Ottawa.

Clinical information of the three index patients

1. LR08-018 (overlap patient)

This previously reported male patient[9] has a number of megalencephaly-capillary malformation (MCAP) syndrome-like features including congenital megalencephaly (with an occipito-frontal circumference of +5.5 standard deviations at 7.5 months), somatic asymmetry, connective tissue dysplasia, and a poorly-substantiated umbilical hemangioma. Serial brain magnetic resonance imaging studies show megalencephaly, mild ventriculomegaly, polymicrogyria and cerebellar tonsillar ectopia. Yet this patient lacked two key MCAP features, namely skin capillary malformations and distal limb anomalies, particularly syndactyly. Using our published diagnostic criteria[9], we considered this patient an overlap patient.

2. LR00-016a1 and the LR00-016 family (MPPH family)

This is a family of European ancestry with features of the megalencephaly-polymicrogyria-polydactyly-hydrocephalus (MPPH) syndrome that consists of four children (one unaffected female, one affected male, and two affected females) and

healthy, non-consanguineous parents. The three affected siblings all had megalencephaly with occipito-frontal circumference of +4-5 standard deviations in their teen years. The affected male (LR00-016a1; one of our index patients) has mild developmental delay particularly involving speech, and was later diagnosed with Asperger syndrome. He underwent third ventriculostomy at 18 months of age because of progressive ventriculomegaly. He has not had any seizures to date. The first affected female (LR00-016a2) has seizures that are well-controlled, as well as mild developmental delay, Asperger-like features and scoliosis. Because of progressive hydrocephalus, two third ventriculostomies were performed but failed, and she underwent ventricular shunting at 13 months of age. The second affected female (LR00-016a3) has intractable seizures and is on multiple anti-epileptic medications, in addition to a vagal nerve stimulator. She has mild developmental problems and has not undergone any neurosurgical shunting procedures.

Brain magnetic resonance imaging studies of the three siblings (performed at 12.5, 12 and 8.5 years, respectively) show similar features of MPPH including striking megalencephaly, bilateral perisylvian polymicrogyria, borderline cerebellar tonsillar ectopia, and a moderately thick corpus callosum. Ventriculomegaly was present in all three siblings and, as noted above, in LR00-016a1 and LR00-016a2 this progressed to hydrocephalus requiring shunting. Of note, none of the three siblings had vascular malformations or digital anomalies such as polydactyly or syndactyly.

The remaining sibling in this family is an older female with a history of mild hearing loss. She has a normal brain magnetic resonance imaging. The mother of all four children underwent a brain magnetic resonance imaging that was normal.

3. LR09-006 (MCAP patient)

This previously reported male patient has classic MCAP features including rapidly progressive megalencephaly[9]. His birth occipito-frontal circumference was +2 standard deviations and progressed to +9-10 standard deviations at 5 years of age. He was large at birth, with somatic asymmetry including limb-length discrepancy, cutis marmorata, bilateral toe syndactyly, and connective tissue dysplasia consisting of skin hyperelasticity, thick subcutaneous tissue and mild ligamentous laxity. He had

progressive hydrocephalus that was shunted at four months of age. Serial brain magnetic resonance imaging studies show bilateral perisylvian polymicrogyria, progressive cerebellar tonsillar ectopia and a thick corpus callosum. This patient also had a small muscular ventricular septal defect, and unilateral microphthalmia managed by enucleation and orbital prosthesis placement.

Cancer and benign tumors reported in MCAP and MPPH

Both MCAP and MPPH demonstrate striking growth dysregulation, which manifests as overgrowth of brain and limbs in both syndromes. Many other tissues are involved, especially vascular, subcutaneous, connective and lymphatic tissue in MCAP and less often in MPPH. In addition, many benign and a few malignant tumors have been reported, which is not surprising as mutations in these genes have been found in a variety of cancers (**Table 1**).

The most common benign tumors in MCAP appear to be vascular, although given the variable terminology used ("cavernous hemangiomas", "angiomata", angiomyolipomas, and "vascular masses"), it is difficult to be certain whether they are true tumors or malformations[6,9-11]. While most common in skin or subcutaneous tissue, several have been described in viscera and in the skull. A few have been complicated by coagulopathy, and one patient had a large tufted angioma of the shoulder complicated by Kasabach-Merritt syndrome[5]. Several patients have had lipomas, and at least one had multiple lipomas[5,9]. Finally, two children have been described with meningiomas, which are rare tumors in this age group (**Supplementary Table 10**).

In contrast, malignant tumors appear to be rare. We found reports of malignancies in only 3 of ~150 (2%) patients with MCAP (5/150 or 3.3% by adding meningiomas), and 1 of ~30 (3.3%) with MPPH (**Supplementary Table 10**). As the MPPH phenotype primarily affects the brain, it is not surprisingly that the tumor involved the brain as well.

Our group has discussed the risk for cancer and the rationale for cancer surveillance in MCAP and MPPH, and we find that the data summarized above is insufficient to make firm recommendations regarding cancer screening in these syndromes. In developing guidelines, we would need to demonstrate an increased tumor

risk in an adequately powered, preferably prospective study with specific inclusion and exclusion criteria. Furthermore, any proposed screening protocol should identify most tumors and provide benefit regarding morbidity and mortality. This has been done for only a few tumor predisposition syndromes. While we have begun a registry for these disorders, prospective studies will require several years to complete.

We already know that several children with MCAP or MPPH have had cancer. While a bias of ascertainment is possible, the reported tumors include two early childhood cancers observed in cancer predisposition syndromes: Wilms tumor (the most common solid tumor of childhood) and medulloblastoma. Further, all three genes and many of these same mutations have been observed repeatedly in many types of sporadic cancer. We therefore need to acknowledge that MCAP and probably MPPH are likely to have a tumor risk higher than the general population. The authors have reached an expert consensus that provisional implementation of a surveillance program is appropriate, and could initially include screening for Wilms tumor – following guidelines developed for Beckwith-Wiedemann syndrome[12] – and brain tumors. Our suggestions for cancer screening are included in the following section on clinical management.

Clinical management

The most frequent medical complications requiring medical management for MCAP and MPPH involve the brain, and include delayed development, seizures, hydrocephalus, cerebellar tonsillar ectopia (including Chiari malformation type 1), and syringomyelia[8,9]. The spectrum of reported symptoms is wide, and the risk for cancer is probably increased. Based on our collective experience to date, we propose provisional management guidelines with the expectation that these will change. At this time, we suggest:

1. Clinic evaluations no less than every 6 months for the first ~6 years of life, and at least yearly thereafter. At each visit, a general medical history should be elicited with attention to the probable increased risk for childhood cancers. In addition, a history of breathing or sleep problems, seizures or other undefined spells, headaches, and behavior changes should be elicited, along with a detailed neurological evaluation.

Any positive history or exam finding should be pursued with appropriate testing, for example sleep studies in individuals with apnea or sleep problems.

2. Baseline brain and spinal cord imaging in all patients with MCAP or MPPH at the time of diagnosis. Based on the limited retrospective data available to date, the risk of hydrocephalus, cerebellar tonsillar ectopia or both with low brainstem or high spinal cord compression appears to be highest in the first 2-4 years of life[8,9]. The risk for brain tumors extends over a longer time period, possibly throughout life (for example, medulloblastoma in young children, and meningiomas in older children and adults). We therefore suggest repeat brain MRI every 6 months from 0-2 years and every year from 2-6 years. In older patients, repeat scans should be performed based on prior brain imaging results and the clinical course, with particular attention to apnea or other abnormal patterns of respiration, headaches, changes in gait or other neurologic problems. Prospective studies are needed to determine appropriate neurosurgical management.
3. Screening for Wilms tumor consisting of renal ultrasounds every 3 months to age 8 years following guidelines for Beckwith-Wiedemann syndrome[12]. Note that AFP is not indicated at this time as this test is directed at early detection of hepatoblastoma, which has not been reported in MCAP or MPPH.
4. Evaluation by a pediatric cardiologist with baseline echocardiogram and electrocardiogram to evaluate for cardiovascular malformations and rhythm abnormalities for all children with MCAP. This suggestion is based on several reports of cardiovascular malformations and rare reports of cardiac rhythm abnormalities[9,13-15], and supported by reports of cardiovascular malformations and rhythm abnormalities in other overgrowth or RASopathy syndromes including Smith-Golabi-Behmel and Costello syndromes[16,17]. Data is insufficient to characterize the natural history of arrhythmia in MCAP and care should be individualized.
5. Baseline thrombophilia evaluation may be warranted as dural sinus stasis and enlargement is common, and thrombosis has been reported in several individuals[14].

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