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Original Article

Sporadic hypothalamic hamartoma is a ciliopathy with somatic and bi-allelic contributions

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Abstract

Hypothalamic hamartoma with gelastic seizures is a well-established cause of drug-resistant epilepsy in early life. The development of novel surgical techniques has permitted the genomic interrogation of hypothalamic hamartoma tissue. This has revealed causative mosaic variants within *GLI3*, *OFD1* and other key regulators of the sonic-hedgehog pathway in a minority of cases. Sonic-hedgehog signalling proteins localize to the cellular organelle primary cilia. We therefore explored the hypothesis that cilia gene variants may underlie hitherto unsolved cases of sporadic hypothalamic hamartoma. We performed high-depth exome sequencing and chromosomal microarray on surgically resected hypothalamic hamartoma tissue and paired leukocyte-derived DNA from 27 patients. We searched for both germline and somatic variants under both dominant and bi-allelic genetic models. In hamartoma-derived DNA of seven patients we identified bi-allelic (one germline, one somatic) variants within one of four cilia genes—*DYNC2I1*, *DYNC2H1*, *IFT140* or *SMO*. In eight patients, we identified single somatic variants in the previously established hypothalamic hamartoma disease genes *GLI3* or *OFD1*. Overall, we established a plausible molecular cause for 15/27 (56%) patients. Here, we expand the genetic architecture beyond single variants within dominant disease genes that cause sporadic hypothalamic hamartoma to bi-allelic (one germline/one somatic) variants, implicate three novel cilia genes and reconceptualize the disorder as a ciliopathy.

Introduction

Hypothalamic hamartoma is a benign congenital lesion of the hypothalamus associated with a well-recognized developmental and epileptic encephalopathy defined by gelastic (laughing) seizures in infancy, which evolve into a drug-resistant epilepsy. Novel surgical techniques have allowed access to these small deep-seated lesions, permitting tissue analysis. Following the discovery that syndromic hypothalamic hamartoma in Pallister Hall syndrome is predominantly caused by germline or *de novo* variants in *GLI3*, a transcriptional regulator of the sonic hedgehog (SHH) pathway (1,2), we and others have demonstrated that sporadic hypothalamic hamartoma can be caused by single somatic variants of *GLI3*, *OFD1* and other genes within the SHH pathway (3–6).

It has also been shown that the SHH pathway proteins localize to the ubiquitous cell organelle, primary cilia, to facilitate cell-to-cell interactions via signal transduction pathways during development (7–12). The importance of this relationship is evidenced by functional studies showing that disruption of cilia genes leads to altered SHH signalling responses during development (8,9,11). Recently,

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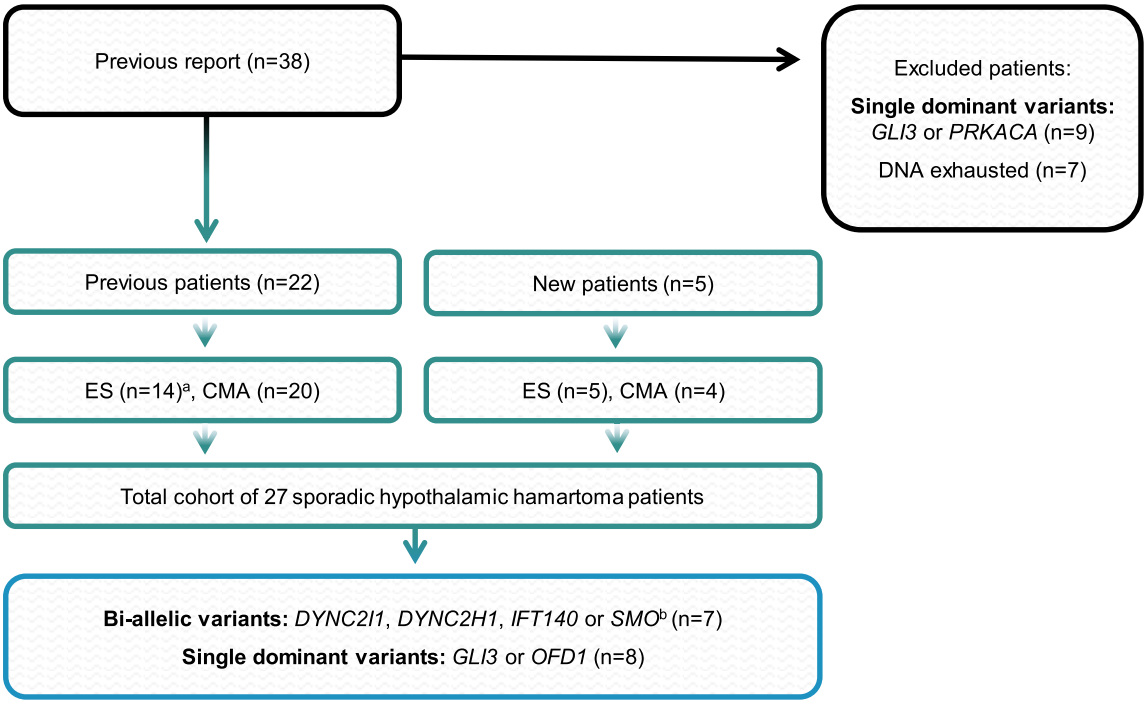

Figure 1. Sporadic hypothalamic hamartoma cohort and genetic analyses. The 27 sporadic hypothalamic hamartoma patients derived from our previously published report (4) and new cases that have not previously been investigated. ES; exome sequencing, CMA; chromosomal microarray. Patients from our previous report with molecular diagnoses ($n = 9$) or insufficient DNA remaining ($n = 7$) for analysis were excluded. [a]Nine patients had exome data re-analysed from our previous study (4). The remaining five underwent exome analysis as part of this study. [b]Three cases had previously identified CNVs that overlap with candidate SHH pathway genes.

the cilia gene *DYNC2H1* [MIM:603297] was implicated in sporadic hypothalamic hamartoma when two Japanese patients with bi-allelic (germline and somatic) variants in hamartoma tissue were described (13).

Here, we systematically analysed a cohort of 27 sporadic patients with hypothalamic hamartoma (Fig. 1) to establish the contribution of cilia gene variants and increase diagnostic yield.

Results

Genetic analyses

Initial analysis of known hypothalamic hamartoma disease genes revealed eight patients with a single somatic variant (Table 1). Four of these patients were found to harbour frameshift variants within the SHH transcription factor *GLI3* [MIM:165240], while the other four (all male) had somatic frameshift variants in the X-linked cilia gene *OFD1* [MIM:300170], including a recurrent variant in two of them. Consistent with these findings, there was a predominance of males (74%) in our cohort (Table 1), as has been observed in other independent hypothalamic hamartoma case series (61%, (14) 64% (15) and 65% (16)), indicating potential under-recognition of *OFD1* or other X-linked genes in the aetiology of hypothalamic hamartoma.

For gene discovery, we then interrogated cilia genes in the remaining 19 unsolved cases and revealed seven cases with confirmed or putative bi-allelic variants, each comprised of a germline and a somatic variant in one of four genes: *DYNC2I1* [MIM:615462], *DYNC2H1*, *IFT140* [MIM:614620] and *SMO* [MIM:601500]. In four cases the variants were confirmed as bi-allelic, whereas in three cases this remained putative (Table 1, Fig. 2).

Three of the seven bi-allelic cases (T1198, T25059, T25063) had distinct germline and somatic variants in *DYNC2I1* that encodes the intermediate chain of the ciliary intraflagellar transport (IFT) dynein-2 complex (Table 1) (17). Two of these cases (T1198, T25063) harboured large somatic copy number variants (CNVs) that encompass *DYNC2I1* on one allele with a novel germline *DYNC2I1* sequence variant on the other; we confirmed these were bi-allelic in hamartoma tissue using both exome and Sanger sequencing (Fig. 2). The third case (T25059) carried two frameshift deletions that were presumed to be bi-allelic, but this could not be confirmed due to the large distance (~22 kb) between the variants precluding allelic discrimination given hamartoma tissue RNA was not available from the patient. Three of the four sequence variants found in *DYNC2I1* were frameshifts resulting in premature stop codons, two of which occur early in the transcript meaning the respective transcripts are likely subject to nonsense-mediated decay, although RNA was unavailable to confirm this. The fourth *DYNC2I1* variant detected was a splice site variant predicted by SpliceAI (18) to alter splicing through disruption of the splice acceptor site at the 5′ end of exon 14 (Δ score = 0.92); tissue RNA was also unavailable from this case.

Table 1. Cases with genetic findings

Case	Sex	ES	CMA	Germline/ Somatic	Gene	RefSeq	Variant/s	AC	SIFT(19)	PP2(20)	Somatic VAF[a]	
											HH	Blood
T322	F	+ (P)	+	Somatic	*GLI3*	NM_000168.6	c.3098del (p.Pro1033ArgfsTer46)	0	N/A	N/A	4/10 (40%)	0/27 (0%)
T1165	M	+ (P)	–	Somatic	*GLI3*		c.3313C > T (p.Gln1105Ter)	0	N/A	N/A	196/522 (38%)	0/208 (0%)
T25069	F	+ (P)	+	Somatic	*GLI3*		c.2977C > T (p.Gln993Ter)	0	N/A	N/A	23/158 (15%)	0/67 (0%)
T1276[c]	M	+ (P)	–	Somatic	*GLI3*		c.3189_3219del (p.Cys1065TrpfsTer4)	0	N/A	N/A	25/139 (18%)	0/48 (0%)
T929[c]	M	+ (P)	+	Somatic	*OFD1*[d]	NM_003611.3	c.702dup (p.Tyr238ValfsTer2)	1	N/A	N/A	80/143 (56%)	0/53 (0%)
T25052	M	+ (P)	+	Somatic	*OFD1*[d]		c.1193_1196del (p.Gln398LeufsTer2)	0	N/A	N/A	5/15 (33%)	0/30 (0%)
T25056	M	+ (P)	+	Somatic	*OFD1*[d]		c.702del (p.Lys237SerfsTer6)	30	N/A	N/A	22/30 (73%)	0/50 (0%)
T25073	M	+ (P)	+	Somatic	*OFD1*[d]		c.702del (p.Lys237SerfsTer6)	30	N/A	N/A	45/80 (56%)	0/41 (0%)
T1198[e]	F	+ (P)	+	Germline & Somatic	*DYNC2I1*/ chr7q11.21-q36.3	NM_018051.5	c.1703-1G > A, chr7q LOH (chr7:58814064–159 138 663)	0, N/A	N/A	N/A	N/A	N/A
T25059	F	+ (P)	+	Germline & Somatic	*DYNC2I1*		c.1083_1083del (p.Asp361ValfsTer2), c.673_676del (p.Lys226ThrfsTer91)	0, 0	N/A	N/A	52/99 (53%)	0/42(0%)
T25063[e]	F	–	+	Germline & Somatic	*DYNC2I1*/ chr7p22.1–q36.3		c.2190_2191del (p.Arg731AlafsTer73), chr7p22.1–q36.3 CNG, CNL (chr7:986211–60 069 242, 58 814 064–159 138 663)	0, N/A	N/A	N/A	N/A	N/A
T25079	M	+ (HH)	+	Germline & Somatic	*DYNC2H1*	NM_001080463.2	c.11186C > A (p.Pro3729His), c.448C > T (p.Arg150Ter)	6, 1	D, N/A	PrD, N/A	15/36 (42%)	N/A
T25080	M	+ (P)	+	Germline & Somatic	*DYNC2H1*		c.8145_8146delinsAT (p.TyrGln2715Ter), c.11437C > T (p.Arg3813Cys)	0, 4	N/A, D	N/A, PrD	89/347 (26%)	0/79 (0%)
T735[e]	F	+ (P)	+	Germline & Somatic	*IFT140*/ chr16p11.2-p13.3	NM_014714.4	c.1901del (p.Lys634ArgfsTer10), chr16p LOH (chr16:0–31 543 619)	0, N/A	N/A	N/A	N/A	N/A
T1094[b]	M	+ (P)	–	Germline & Somatic	*SMO*	NM_005631.5	c.1453C > G (p.Arg485Gly), c.1274_1275del (p.Leu426ValfsTer13)	0, 1	D, N/A	D, N/A	135/400 (34%)	0/87 (0%)

ES; exome sequencing, CMA; chromosomal microarray, AC; gnomAD allele count, PP2; PolyPhen2, (HH); hypothalamic hamartoma only, (P); paired blood and hypothalamic hamartoma tissue, LOH; loss of heterozygosity, CNG; copy number gain, CNL; copy number loss, N/A; not applicable, D; damaging, PrD; probably damaging [a]Variant allele fraction; Variant reads/Total reads [b]Confirmed bi-allelic variants [c]Not validated with Sanger sequencing or ddPCR due to insufficient DNA [d]X-linked gene, hence mosaic VAF can be >50% in hemizygous males [e]CNV Identified in previous analyses (4)

Figure 2. The exome and Sanger sequencing data supporting a bi-allelic allelic phase in the three cases T1198, T25063 and T735. In all three cases, the corresponding germline variant is the most prevalent allele in hypothalamic hamartoma tissue indicating the somatic CNVs detected in these cases are on the alternate allele. (**A**) Integrative genomics viewer (IGV) shot of paired exome sequencing of T1198 showing the *DYNC2I1* c.1703-1G > A variant, top panel within IGV is hypothalamic hamartoma and bottom panel is blood. (**B**) IGV shot of paired exome sequencing of T735 showing the *IFT140* c.1901del; p.Lys634ArgfsTer10 variant, top panel within IGV is hypothalamic hamartoma and bottom panel is blood. (**C**) Sanger sequencing of *DYNC2I1* c.2190_2191delAA; p. Arg731AlafsTer73 variant within T25063.

Two cases (T25079, T25080) were found to have germline and somatic variants in *DYNC2H1* that encodes the ciliary heavy chain IFT motor protein dynein-2 (Table 1). In these two cases, we identified protein-truncating variants (PTVs) (nonsense or frameshift) in conjunction with missense variants that are predicted damaging by *in silico* tools (19,20). The large genomic distances (189 and 108 kb) between these respective sequence variants precluded phasing in the absence of hamartoma tissue RNA.

We also identified one patient (T735) with bi-allelic variants in the *IFT140* gene encoding one of six sub-units of the IFT-A protein complex (Table 1) (12). This patient harboured a large somatic CNV encompassing

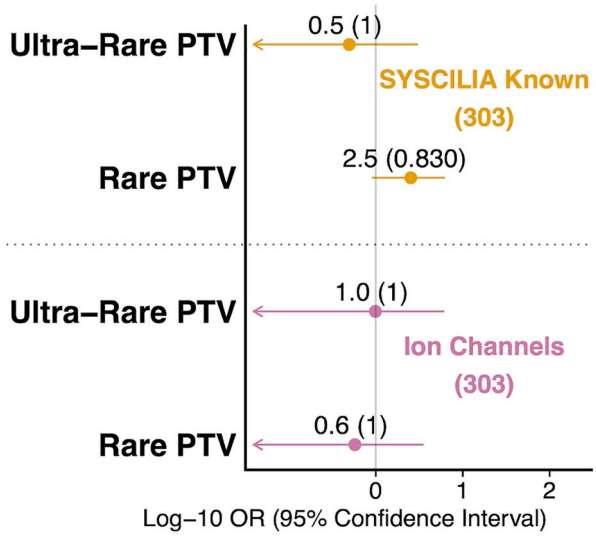

Figure 3. Exploring burden of protein truncating variants in hypothalamic hamartoma patients in two gene sets separated by minor allele frequency. The two gene sets listed are known cilia genes and ion channel genes. 'Ultra-rare' refers to variants absent in gnomAD, 'Rare' refers to variants present in gnomAD but with a population-specific minor allele frequency less than 0.1%. 'PTV' indicates protein truncating variant. Number of genes in set in parenthesis. Odds ratio, confidence intervals and FDR corrected *p*-value were generated from the Fisher's exact test. Odds ratio is displayed for each test with FDR-adjusted *p*-values in parentheses. X-axis displays the log of the odds ratio and confidence intervals. Arrows indicate lower bound of confidence interval has been truncated.

IFT140 in combination with a novel germline frameshift variant that introduces a premature stop codon. As this variant occurs in the last nucleotide of exon 16 it is predicted to result in loss of the consensus donor splice site by SpliceAI (Δ score = 1.00). A *trans* configuration in hamartoma tissue for these variants was confirmed on sequencing (Fig. 2).

The seventh case (T1094) had bi-allelic variants in the cilia gene *SMO* that encodes a frizzled class receptor within the SHH pathway known to be trafficked and signal within the primary cilium (Table 1) (7). In this patient, we identified a novel missense germline variant in combination with a somatic frameshift variant introducing a premature stop codon that may also lead to nonsense-mediated decay. Due to the close genomic proximity between these two variants, we were able to perform allelic discrimination by restriction digest, which confirmed these two variants were in a *trans* configuration (Supplementary Material, Fig. S1).

Gene enrichment analysis

Given the above discoveries, and in the absence of further analysable tissue samples, we sought alternate indirect evidence for a possible bi-allelic mechanism involving cilia genes. We posited there may be an excess of potentially damaging rare germline variants in cilia genes (detectable in blood) within sporadic hypothalamic hamartoma cases. We therefore sought to quantify the presence of rare germline qualifying variants with a population-specific minor allele frequency <0.1% in sporadic hypothalamic hamartoma patients compared with 2413 controls across four different gene-sets (Supplementary Material). In order to assess these germline contributions, we also included an additional 25 sporadic hypothalamic hamartoma cases on which we had access to exome data generated from blood-derived DNA.

Given the recessive inheritance of most ciliopathies (21), we reasoned that the germline variants would also be present in the general population. PTVs in known cilia genes which showed a trend towards enrichment were further divided into ultra-rare (absent in gnomAD) and rare (present in gnomAD but population-specific allele frequency <0.1%) with ion channel genes as a control (Fig. 3). Among PTVs in known cilia genes, PTVs were primarily rare ($OR = 2.5$, $CI = [0.89–6.2]$, $p = 0.039$, $adj.p = 0.83$) and not ultra-rare ($OR = 0.5$, $CI = [0.0–3.0]$, $p = 0.72$, $adj.p = 1.0$), which is consistent with our hypothesis (Fig. 3). Despite no statistically significant association being found, our data revealed a trend that could be strengthened in future by increasing the size of our cohort (Supplementary Material, Fig. S6). Additionally, as ciliopathies are generally recessively inherited, our findings may reflect the weak selection pressure on deleterious heterozygous germline variants as has been demonstrated with disease genes for disorders with autosomal recessive inheritance (21,22).

Discussion

We have expanded the molecular architecture of sporadic hypothalamic hamartoma by implicating three new cilia genes (*DYNC2I1*, *IFT140*, *SMO*) involved in intraflagellar transport (IFT), and replicated previous findings (13) of bi-allelic *DYNC2H1* variants. Our findings of confirmed or putative bi-allelic variants within a single gene comprising of a germline and somatic variant are analogous to other reported brain lesions that can cause epilepsy including subependymal giant cell astrocytomas, cortical tubers in tuberous sclerosis complex and focal cortical dysplasias (23–25).

Primary cilia comprise a single non-motile axoneme that is constructed and maintained by protein complexes known as the IFT machinery (12). The IFT machinery consists of two protein complexes, IFT-A and IFT-B, which are required for retrograde and anterograde transport, respectively (12). It has been demonstrated in numerous studies that SHH pathway constituents localize to primary cilia and are dependent upon IFT machinery for signal transduction (7–10,26). *DYNC2H1* and *DYNC2I1* encode subunits of the IFT dynein-2 molecular motor together with *DYNLT2B* [MIM:617353], *DYNC2LI1* [MIM:617083] and *DYNC2I2* [MIM:613363] (12). This dynein-2 motor drives the IFT-A protein complex, of which *IFT140* is a subunit, to facilitate retrograde transport (Fig. 4) (12).

'Ciliopathies' is a term applied to a heterogeneous group of disorders, typically recessively inherited with

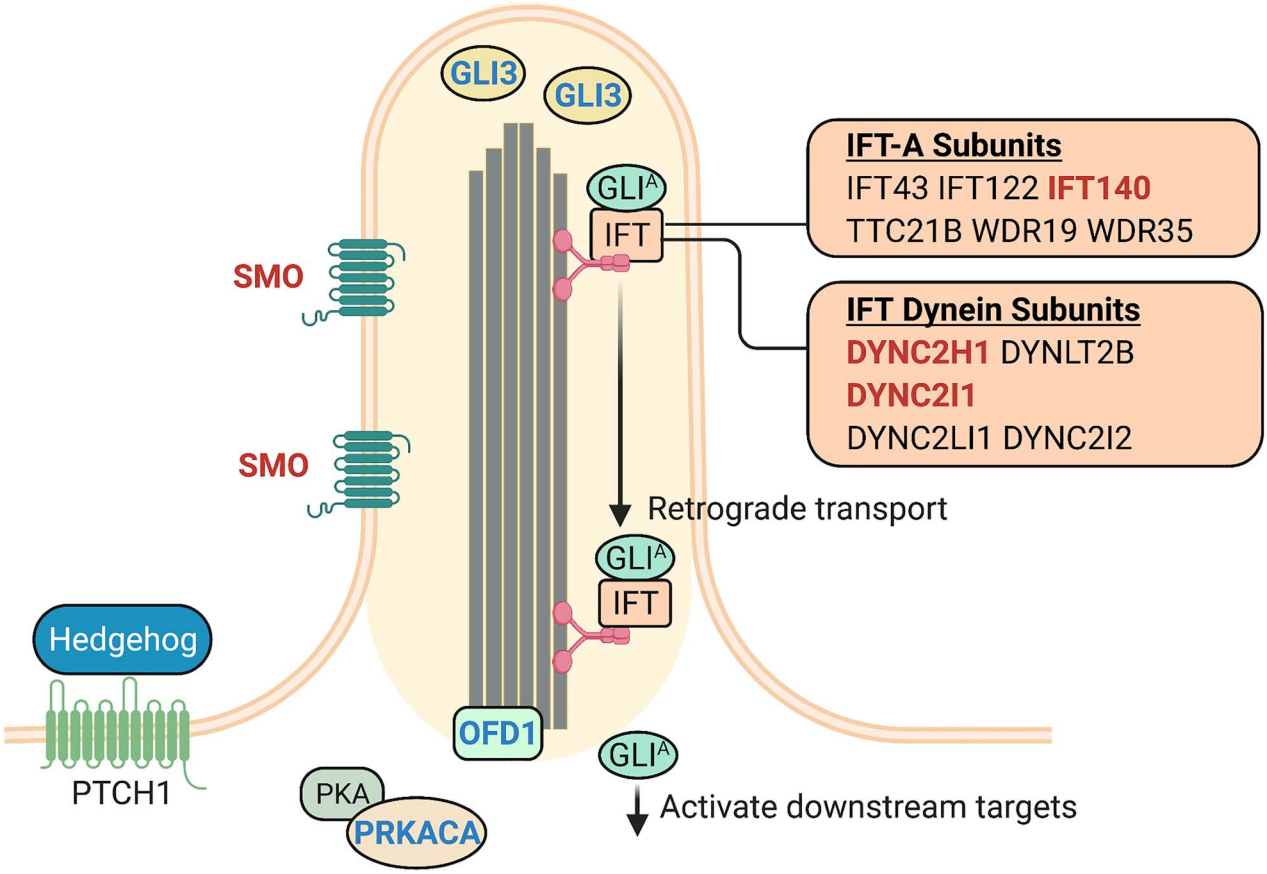

Figure 4. The sonic hedgehog pathway and primary cilia. In response to the hedgehog ligand binding to PTCH1, SMO localizes to the axoneme of primary cilia (7). The GLI protein complexes including GLI3 localize to the tip of primary cilia (8) and are processed into the activated form, GLI^A. IFT mediated retrograde transport and specifically the IFT dynein-2 and IFT-A complexes facilitate the movement of the GLI^A complex to regulate downstream targets of the pathway. Red text; the genes in which bi-allelic variants have been identified within our sporadic hypothalamic hamartoma cohort. Blue text; the genes in which dominant somatic variants have previously been identified in sporadic hypothalamic hamartoma. *OFD1* encodes a protein that localizes to the basal body of primary cilia and is required for ciliogenesis (12). Single somatic *PRKACA* variants have previously been identified in sporadic hypothalamic hamartoma (4) which encodes one of two catalytic subunits of the PKA protein complex that localizes to primary cilia (42). Figure adapted from (12).

germline variants in cilia genes causing disruption of ciliogenesis or IFT (21). Interestingly, bi-allelic *germline* variants in the *DYNC2H1*, *DYNC2I1* and *IFT140* genes are associated with the related skeletal ciliopathies Jeune Asphyxiating Thoracic Dystrophy (JATD [MIM: 208500]), Short Rib Polydactyly (SRPS [MIM: 615503]) and Mainzer–Saldino (MSS [MIM 266920]) syndromes (27–30). Functional interrogation of bi-allelic germline variants within *DYNC2H1*, *DYNC2I1* or *IFT140* has demonstrated disruption of retrograde trafficking and ciliogenesis within primary cilia (27,29,30). The lack of skeletal features in our cases is presumably explained by the brain-specific somatic second hit. Notably, hypothalamic hamartoma has not been reported in these ciliopathies with two *germline* variants. This may represent disease-specific genotype–phenotype associations, similar to those well established for Pallister–Hall and Greig Cephalopolysyndactyly syndromes due to pathogenic *GLI3* variants (2). We observe that bi-allelic variants identified in patients with hypothalamic hamartoma may have more severe impact. For example, patients we report with *DYNC2I1*, *IFT140* and *SMO* bi-allelic variants have one PTV in combination with either a second PTV or loss of heterozygosity (LOH) copy number variant (CNV) which may severely disrupt the protein (Table 1). In contrast, germline bi-allelic variants in *DYNC2I1* and *IFT140* associated with ciliopathies tend to include a combination of a potentially less damaging missense variant and a PTV (28–31). The recent identification of germline *SMO* variants has demonstrated that patients with hypothalamic hamartoma have at least one PTV as opposed to patients without hypothalamic hamartoma carrying only missense variants (32,33). However, these observations are based on only small numbers and further cases and functional analyses of variants is required to establish any mutational spectrum and genotype–phenotype associations.

Our data implicate genes involved in IFT-mediated retrograde trafficking in primary cilia in the molecular pathogenesis of sporadic hypothalamic hamartoma. Notably, the pathological mechanisms underlying formation of hypothalamic hamartomas remain unclear even for the established *GLI3* variants (5). For example,

the *GLI3* mouse model of Pallister–Hall syndrome does not develop hypothalamic hamartomas (34). Disruption of SHH pathway signalling appears to be central to the pathogenesis. With respect to this, the cilia genes (*OFD1*, *DYNC2I1*, *DYNC2H1*, *IFT140* or *SMO*) identified in this study have all been shown to disrupt SHH pathway proteins and lead to aberrant SHH signalling in prior studies (5,30,33,35,36). We propose that dysfunction of specific components of the IFT machinery localized to hamartoma cells interferes with normal SHH signalling, a mechanism which will require functional interrogation in future studies. Consistent with this, we implicated a third cilia gene in sporadic hypothalamic hamartoma, Smoothened (*SMO*), which is also a regulator of the SHH pathway. It encodes a seven-transmembrane protein that localizes to primary cilia and depends on IFT to regulate SHH signalling during development (Fig. 4) (7,26). Moreover, *germline SMO* bi-allelic variants have recently been reported in five individuals with syndromic hypothalamic hamartoma and other developmental anomalies including post-axial polydactyly, microcephaly and skeletal abnormalities that often present in the aforementioned skeletal ciliopathies (32,33,37).

In summary, we have identified plausible molecular causes on a research basis in 15/27 (56%) cases in our sporadic cohort including confirmed or putative single somatic or bi-allelic variants. Taken together, our results suggest that disrupted trafficking and SHH signalling in the primary cilium underlie the pathogenesis of sporadic hypothalamic hamartoma through two distinct genetic mechanisms. Firstly, through single somatic variants in disease genes such as *GLI3* and *OFD1* that function within primary cilia during development, a mechanism that we and others previously established (3–6). Secondly, through bi-allelic variants in cilia genes disrupting IFT and SHH signalling, suggesting we can reconceptualize sporadic hypothalamic hamartoma as a ciliopathy.

Materials and Methods

Patient cohort and sample collection

We studied resected hypothalamic hamartoma tissue from 22 previously reported unsolved (4) and five newly recruited patients with sporadic disease (Fig. 1), comprising 20 (74%) males and seven (26%) females. Epilepsy commenced in the first year of life for 22/27 of these patients, and all had gelastic seizures. Additional features included intellectual disability ($n = 20$) and central precocious puberty ($n = 11$) (Supplementary Material, Table S1). None had additional syndromic features of digital, oro-facial abnormalities or visceral malformations, and none had a family history of hypothalamic hamartoma. An additional 25 sporadic hypothalamic hamartoma cases with only blood-derived DNA available were studied, exclusively in our gene enrichment analysis.

DNA was extracted from fresh frozen or formalin-fixed paraffin-embedded hamartoma tissue, obtained during surgical treatment of hamartomas, and from whole-blood using Qiagen All Prep DNA/RNA, FFPE Tissue and QIAamp DNA Blood Maxi Kits (Hilden, Germany), respectively, and previously reported standard protocols (4). The Human Research Ethics Committees of Austin Health and The Royal Children's Hospital in Melbourne and the Institutional Review Board of St. Joseph's Hospital and Medical Center in Phoenix approved this study. Informed consent was obtained from affected individuals or their parents or legal guardians in the case of minors, those with intellectual disability or deceased individuals.

Exome sequencing

There was insufficient hamartoma-derived DNA to perform exome sequencing and chromosomal microarray (CMA) analysis on all cases. Exome sequencing was possible on 19/27 patients including 18 with paired hamartoma/blood-derived DNA and one with only hamartoma-derived DNA (Fig. 1). Exome sequencing was performed using the Agilent SureSelect DNA Human All Exon V6, 96RXN kit (Agilent Technologies, Santa Clara, CA) and the Illumina NovaSeq 6000 System (Illumina, San Diego, CA, USA) or the Illumina TruSeq Exome Enrichment kit and HiSeq 2000 System (Illumina, San Diego, CA, USA). Briefly, reads were aligned to the hg19 reference genome with BWA-MEM v0.7.17-r1188, then duplicate marking and base quality score recalibration performed with the Genome Analysis Toolkit (GATK). Germline variant calling was performed with GATK HaplotypeCaller and somatic variant calling with GATK Mutect2 v4.0.1.2, VarScan v2.4.3 and Strelka v2.9.10. Variants were annotated using vcfanno and ANNOVAR.

We performed a targeted search for germline and somatic variants in a curated list of known or candidate cilia and SHH pathway genes compiled from the SYSCILIA Gold Standard (38) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (39) catalogues. Variants were filtered according to the following criteria: located in a coding or splice site region, frequency of less than or equal to 0.001 in the Genome Aggregation Database (gnomAD v2.1.1) (40), and variant type—missense, nonsense, coding indel or splice site.

CMA analysis

CMA data were generated from 24 cases, 20/24 of these as part of our previous study (Fig. 1) (4). For the four new cases, the Illumina Global Diversity array platform (GDAv1.0 San Diego, CA) was used to perform genome-wide CNVs and LOH screening at a resolution of 200 Kb and 2 Mb, respectively, on hamartoma and blood-derived DNA. The data were analysed using NxClinicalv6.0 (BioDicovery, CA) using genome reference sequence NCBI37/hg19.

PCR and Sanger sequencing

Amplification was performed using gene-specific primers (available on request) designed to reference human gene transcripts using NCBI Gene (https://www.ncbi.

nlm.nih.gov/gene) and Primer3 v4.1.0 (http://bioinfo.ut.ee/primer3/). Amplification reactions were cycled using a standard protocol on a Veriti Thermal Cycler (Applied Biosystems, Carlsbad, CA) and sequencing performed with a BigDyeTM v3.1 Terminator Cycle Sequencing Kit (Applied Biosystems). Sequencing products were resolved using a 3730 XL DNA Analyzer (Applied Biosystems). All nucleotide changes were called using Codon Code Aligner software (CodonCode Corporation, Dedham, MA).

Droplet digital PCR

Custom probes and primers were ordered from Integrated DNA Technologies, Inc. (Iowa, USA) and were utilized to validate and quantify somatic variants. Droplet generation, PCR cycling and droplet reading were performed according to the manufacturer's recommendations (Bio-Rad, Hercules, CA). Briefly, probes and primers were mixed with 2x ddPCR Supermix (No dUTPs) for probe (Bio-Rad) at 250 and 900 nM final concentrations for each probe and each of the primers, respectively, and mixed with 10 ng of DNA sample to a final volume of 23 μL. Twenty microliters of reactions were loaded in an eight-channel droplet generator cartridge (Bio-Rad) and droplets were generated with 70 μL of droplet generation oil (Bio-Rad) using the manual QX200 Droplet Generator. Following droplet generation, samples were manually transferred to a 96-well PCR plate, heat-sealed and amplified on a C1000 Touch thermal cycler using the following cycling conditions: 95°C for 10 min for one cycle, followed by 40 cycles at 94°C for 30 s and 55°C for 60 s, one cycle at 98°C for 10 min and 4°C hold. Post-PCR products were read on the QX200 droplet reader (Bio-Rad) and analysed using the QuantaSoft software.

Allelic discrimination by restriction digestion

Two restriction enzymes, FastDigest PfoI and TaaI (Thermo Scientific™, Waltham, MA), were utilized according to the manufacturer's recommendations in a 30 μL reaction volume to sequentially differentiate variant alleles. Restriction digest reactions were resolved with standard agarose gels and visualized under UV light with a UVP GelDoc-It310 imaging system. Digested fragments were gel extracted using the Qiagen QIAquick Gel Extraction Kit (Hilden, Germany) as per the manufacturer's instructions (Supplementary Material).

Gene enrichment analysis

Gene enrichment analysis was performed as previously described (41) by quantifying ultra-rare germline variants in sporadic patients with hypothalamic hamartoma and 2413 ethnically matched population controls using four different gene-sets: SHH pathway genes (49 genes) from the KEGG database, and known cilia genes (303 genes) and potential cilia genes (410 genes) from SYSCILIA and 303 randomly selected ion channel genes from the HGNC database given the lack of known overlap between these proteins and hypothalamic hamartoma aetiology (Supplementary Material) (38,39,42).

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. The authors have no conflicts of interest to declare.

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Author Contribution

M.S.H. and S.F.B developed the idea and directed the project. T.E.G., Z.Y., N.G.G., J.A.D., H.M., B.W.D., E.L.H. and M.S.H. performed molecular genetics experiments. N.G.G., J.M., M.F.B., M.B., A.B and E.L.H. performed

bioinformatics analysis. E.J.C., R.B., R.J.L. A.S.H., J.L.F., P.J.L., L.G.S., I.E.S., J.F.K. and S.F.B. conducted clinical phenotyping or specimen collection. M.S.H., R.J.L., P.J.L., D.B.G., E.L.H. and S.F.B. provided equipment and reagents. T.E.G., S.F.B. and M.S.H. drafted the paper. All authors discussed the results and commented on the final manuscript.

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