



HHS Public Access

Author manuscript

Genet Med. Author manuscript; available in PMC 2018 October 20.

Published in final edited form as:

Genet Med. 2018 November ; 20(11): 1354–1364. doi:10.1038/gim.2018.8.

Analysis of 17 genes detects mutations in 81% of 811 patients with lissencephaly

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Conflict of interest notification page

None of the authors have any competing interests. The content is solely the responsibility of the authors, and does not necessarily represent the official views of the National Institutes of Health. The funding sources had no role in the design and conduct of the study, collection, management, analysis and interpretation of the data, preparation, review or approval of the manuscript, or decision to submit the manuscript for publication.

Author Contributions

NDD, RG, and WBD designed the study, analyzed the data and drafted the manuscript; AET analyzed the data and drafted a portion of the manuscript; KAA, GMM, JTN, SC, CO, DM, SC, GG, CTM, JBR, MSZ, JGG, AR, VC, EP, DHL acquired and analyzed clinical, imaging and molecular data. All authors read, edited and approved the final draft.

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Abstract

Purpose—To estimate diagnostic yield and genotype-phenotype correlations in a cohort of 811 patients with lissencephaly or subcortical band heterotopia.

Methods—We collected DNA from 756 children with lissencephaly over 30 years. Many were tested for deletion 17p13.3 and mutations of *LIS1*, *DCX* and *ARX*, but few other genes. Among those tested, 216 remained unsolved and were tested by a targeted panel of 17 genes (*ACTB*, *ACTG1*, *ARX*, *CRADD*, *DCX*, *LIS1*, *TUBA1A*, *TUBA8*, *TUBB2B*, *TUBB*, *TUBB3*, *TUBG1*, *KIF2A*, *KIF5C*, *DYNC1H1*, *RELN* and *VLDLR*) or by whole exome sequencing. 55 patients studied in another institution were added as a validation cohort.

Results—The overall mutation frequency in the entire cohort was 81%. *LIS1* accounted for 40% of patients, followed by *DCX* (23%), *TUBA1A* (5%), and *DYNC1H1* (3%). Other genes accounted for 1% or less of patients. 19% remained unsolved, which suggests that several additional genes remain to be discovered. The majority of unsolved patients had posterior pachygyria, subcortical band heterotopia or mild frontal pachygyria.

Conclusions—The brain-imaging pattern correlates with mutations in single lissencephaly-associated genes, as well as in biological pathways. We propose the first LIS classification system based on the underlying molecular mechanisms.

Keywords

lissencephaly; subcortical band heterotopia; tubulinopathy; actinopathy; reelinopathy

INTRODUCTION

Lissencephaly (LIS) comprises a spectrum of malformations of cortical development caused by deficient neuronal migration with one notable exception related to deficient apoptosis. The LIS spectrum includes agyria defined as cortical regions with sulci >3 cm apart, pachygyria defined as abnormally wide gyri with sulci 1.5-3 cm apart, and subcortical band heterotopia (SBH) defined as longitudinal bands of gray matter located deep to the cerebral cortex and separated from it by a thin layer of white matter.¹ The cerebral cortex in agyria and pachygyria can be either very thick (10-20 mm in thick or classic LIS), or less often only mildly thick (5-10 mm in “thin” LIS). We recently revised our LIS classification system to allow more reliable prediction of the most likely causative genes using brain-imaging patterns.¹

All patients with LIS have intellectual disability, but the severity differs significantly based on the subtype of LIS from profound disability and limited survival with diffuse agyria to mild intellectual or learning disability in patients with partial SBH. Seizures occur in most patients with LIS.^{1,2}

LIS has a strong genetic basis.² To date 20 genes have been associated with LIS (Figure 1, and Supplementary Table 1 with references). Several studies have examined mutation

frequency and genotype-phenotype correlation for *LIS1*, *DCX* and *TUBA1A*.³⁻⁷ However, 11 of 20 LIS-associated genes have been discovered during the past six years, so little data is available regarding the prevalence of mutations and genotype-phenotype correlations. An accurate estimation of the contributions of different genes to LIS has proven difficult,² with most data coming from studies of specific subtypes of LIS or all malformations of cortical development combined.⁸⁻¹⁰

Here we report results of targeted sequencing of 17 LIS causative genes in 216 children with unexplained LIS, and integrate these with prior results from our 30-year cohort of 988 LIS patients with any testing data available. These results are compared with the specific imaging patterns observed using an expanded imaging classification system.¹

MATERIALS AND METHODS

Subjects

Our Seattle-based research database contains information on ~1400 individuals with LIS. We excluded another ~60 patients with MLIS defined as birth OFC more than 3 SD below the mean, as this group is much less well characterized with only rare causative genes known (i.e. *KATNB1*, *NDE1*, *RNU4ATAC*). Results of previous genetic testing were available for 988 patients, including 540 positive and 448 negative results (Figure 1). Most unsolved patients were tested for mutations in *LIS1* and *DCX*, and some for mutations in *ARX*, *TUBA1A*, *ACTB* or *ACTG1*. We had in hand DNA samples from 216 unsolved patients including 193 sporadic subjects and 10 multiplex families. From this group, testing was negative for *LIS1* including deletion 17p13.3 in 109, *DCX* in 101, and *TUBA1A* in 67 patients, while 72 (33%) had no prior genetic testing. The remaining 232 patients with incomplete testing and no DNA sample were excluded, leaving us with a primary LIS study cohort of 756 patients. We included another 55 patients studied at A. Meyer Children's Hospital in Florence, Italy as a validation cohort. Institutional review boards at The University of Chicago, Seattle Children's Hospital, and Meyer Children's Hospital approved this study.

Historical Results

About 75% of positive tests were performed in the Ledbetter (prior to 2004) or Dobyns (2004 onward) labs, and about 25% at outside labs. The tests included chromosome analysis, fluorescence *in situ* hybridization for deletion 17p13.3, chromosome microarrays, MLPA for *LIS1*, *DCX* and *RELN*, Sanger sequencing of single genes (*LIS1*, *DCX*, *ARX*, *TUBA1A*, *TUBB2B*, *RELN*, *VLDLR*, *ACTB* and *ACTG1*), and whole exome sequencing (WES).

Sequencing

We designed a targeted sequencing panel of 17 genes (Figure 1), using single molecule molecular inversion probes (smMIPs).¹¹ We excluded *CDK5* as this report appeared after our study was underway,¹² and excluded microlissencephaly genes (*KATNB1*, *NDE1*, *RNU4ATAC*) as our cohort did not include patients with matching phenotypes.¹³⁻¹⁵ Massively parallel sequencing was performed on Illumina HiSeq and MiSeq platforms. Seven patients were studied using WES alone or with smMIPs (Supplementary Methods).

Our Florence cohort was studied using a custom designed targeted panel that included all 17 genes.

Assessment of pathogenicity

Variant interpretation was done using American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology (AMP) guidelines, except that we analyzed only variants with allele frequencies <1% in public databases.¹⁶

Confirmation

Constitutional and mosaic point mutations were confirmed by Sanger sequencing. Copy number variants were validated using qPCR. Parental samples were available for 70 patients and used for segregation analysis with Sanger sequencing and qPCR. We also used Sanger sequencing for regions with low coverage in the *ACTB* and *ACTG1* genes in patients with frontal-predominant pachygyria. We did not test other low coverage genes in this cohort.

RESULTS

Targeted sequencing and WES identified (likely) pathogenic variants in 74 of 216 (34%) patients (Supplementary Table 1). The total number of mutations and patterns of LIS associated with each gene are shown in Table 2. Detailed molecular and clinical data for mutation positive patients are shown in Supplementary Tables 2 and 3. Mutations of *LIS1* or *DCX* were found in 23 patients in whom prior testing had not been completed. We considered 12 variants to be causative even though ACMG-AMP criteria for pathogenicity classify them as variants of unknown significance (VOUS), mostly due to lack of parental samples. However, consistent imaging phenotypes argue strongly for causality of the variants detected, and we believe that all are in fact pathogenic.

Mutation distribution in our entire cohort and validation cohort

We next collated data on mutations detected in 540 subjects prior to this study, and combined them with results of our smMIP sequencing panel to show mutations in 614 of 756 (81%) patients (Figure 2). Given the long accrual time and different testing methods used, these results could reflect ascertainment and testing bias. To address this question, we compared data in the full cohort with more recent data from Seattle (Dobyns lab) and from the A Meyer Children's Hospital in Florence, Italy (Guerrini lab), finding mutations in 59 of 70 (84%) and 41 of 55 (76%) patients, respectively (Figure 2B). Notably, the mutation frequencies closely overlap across all three cohorts, including the fraction of patients with no mutations detected. The most commonly mutated genes across all three cohorts were *LIS1*, *DCX*, *DYNC1H1* and *TUBA1A*. We found a higher proportion of more severe phenotypes (i.e. mutations in *TUBA1A*) and more multiple congenital anomaly syndromes (i.e. mutations in *ARX*, *ACTB* or *ACTG1*) in the Dobyns cohort.

Diagnostic yield

We detected causative mutations in 655 of 811 (81%) patients in our combined LIS cohort, and found that mutations of four genes – *LIS1*, *DCX*, *TUBA1A* and *DYNC1H1* in this order – account for 571 of 811 (70.5%) individuals with LIS. The number of children with severe

ARX mutations is also high, likely the result of active recruitment. All other LIS genes had frequencies of ~1% or less. Even with testing of 17 LIS genes, about 20% of patients remain unsolved. While some may have undetected mutations of known LIS genes, we expect that most have mutations of as yet unknown genes.

Unsolved phenotypes

As expected, the rate of detecting mutations differed significantly between phenotype groups. We found pathogenic mutations in 97% of children with diffuse agyria, 95% with diffuse SBH, 92% with posterior-predominant mixed agyria-pachygyria (mostly *LIS1*), 72-75% with diffuse pachygyria (anterior- or posterior-predominant), and 65% with imaging findings suggestive of tubulinopathies (Table 1). Thus, several and possibly many more classic LIS and SBH genes have yet to be identified. For example, a homozygous truncating mutation of *CDK5* was just reported in 4 children with severe LIS and cerebellar hypoplasia from a large consanguineous family.¹²

We identified mutations in posterior predominant SBH in only 2 of 15 (13%) patients, both with mosaic *LIS1* mutations. A recent report described both germline and mosaic mutations of *STX7* in a family with three affected individuals.¹⁷ And we found *CRADD* mutations in 6 of 20 individuals with the newest subtype of LIS – anterior predominant thin (undulating) LIS with normal cerebellum.¹¹

Biological network based classification

We next reversed the axes of Table 1 to organize LIS phenotypes based on their associated genes, and separated the 20 genes into 8 groups based on shared intracellular localization, protein function and, whenever possible, shared LIS pattern on brain imaging, a process that was facilitated by our recent analysis of LIS phenotypes (Table 2).¹ For some groups, the shared function was obvious such as microtubule structural proteins (alpha and beta tubulins), and the Reelin signaling pathway (*RELN*, *VLDLR*).

The most difficult proved to be the 9 microtubule-associated proteins (MAPs), as several have multiple functions in the cell, especially *LIS1* and *DCX*, and the imaging patterns differed. We placed *TUBG1* and *LIS1* (plus the *LIS1* modifier *YWHAE*) in a centrosome-associated MAP group as both are highly expressed at the centrosome and share the same posterior predominant thick LIS pattern, especially at the mild end of the *LIS1* spectrum. *NDE1* is also expressed at the centrosome. The phenotype is more severe than *LIS1* because of severe congenital microcephaly, but requires biallelic mutations. In mouse, biallelic *Lis1* mutations with 35% residual *Lis1* expression also result in microcephaly.¹⁸ We placed the two actin structural proteins in an actin-based group, and noted that both are associated with frontal predominant thick LIS. This same uncommon pattern occurs with mild mutations of *DCX*. However, we expanded this group to include *DCX* only after several reports showed that *DCX*, while also interacting with microtubules, functions to regulate the actin cytoskeleton.^{19,20} Of course, the placement of these genes will need to be re-visited as knowledge increases.

DISCUSSION

Our work represents the first systematic study of a nearly complete set of known causal genes in a large well-studied LIS cohort, finding mutations in 74 of 216 (35%) unexplained and partially pretested LIS patients (Table 1) by targeted or WES sequencing. Our genetic testing results allow detailed genotype-phenotype analysis for individual genes as well as gene groups. In the following sections we discuss the mutation frequency and imaging patterns seen with individual genes and groups, and provide further review of previous studies and comments regarding mosaicism in the Supplementary Discussion.

Centrosome-expressed microtubule associated proteins (MAPs)

This model group includes the *LIS1*, *YWHAE*, *TUBG1* and *NDE1* genes. Despite the diversity of *LIS1* functions, we classified *LIS1* here based on phenotypic overlap with *TUBG1*. With no exceptions, mutations in *LIS1* and *TUBG1* cause posterior predominant thick LIS. Deletions of both *LIS1* and *YWHAE* cause near complete agyria with no clear gradient. Non-cortical malformations were uncommon and usually not severe, except for *NDE1*.

We found heterozygous deletions or mutations of *LIS1* in 322 of 811 (40%) patients, making this gene by far the most frequent cause of LIS (Table 2). The most common class of *LIS1* mutations all result in loss-of-function (n=21 of 23 found in panel sequencing). The phenotype almost always consists of mixed anterior pachygyria and posterior agyria.

Mutations of *LIS1* have also been found in posterior-predominant (never diffuse) SBH. These are usually mosaic mutations with intermediate levels of mosaicism (allele frequency ~30%),⁶ although we have identified two patients with apparent germline mutations. Remarkably, one of the mosaic mutations associated with partial posterior SBH (Arg273*) was previously reported as a constitutional mutation in patients with severe LIS.²¹ This observation supports the expectation that mosaic mutations of *LIS1* result in a less severe SBH phenotype.

Heterozygous mutations of *TUBG1* were detected in only 6 of 811 (0.7%) patients, and consisted of two novel substitutions, Ile257Phe and Ser259Leu. The latter was recurrent, seen in 5 of 6 patients including two siblings. Sequencing in blood-derived DNA was negative in both parents, indicating germline mosaicism. Brain imaging in all 6 patients has shown the same posterior predominant pattern of LIS seen with *LIS1* mutations, especially less severe *LIS1* missense mutations. None of the patients in our cohort demonstrated the severe microcephaly previously reported with *TUBG1* mutations.⁸

Tubulinopathies (TUBs)

To date genes encoding two alpha protein isoforms (*TUBA1A* and *TUBA8*) and three beta-tubulin isoforms (*TUBB2B*, *TUBB* and *TUBB3*) have been associated with malformations of cortical development, variously classified as LIS, polymicrogyria, polymicrogyria-like or most recently as tubulinopathy-associated dysgyria.^{1,4,10,22} We identified mutations in all LIS-associated tubulin genes except for *TUBA8*, which to date have been reported in only two distantly related consanguineous families.²³

Mutations of *TUBA1A* accounted for ~5% of LIS patients, making it the third most frequent gene in the Dobyns cohort. Among 38 patients with LIS and *TUBA1A* mutations, we found lissencephaly with cerebellar hypoplasia (LCH) associated with congenital microcephaly and usually thin cortex (14%), LCH with borderline microcephaly or normal head size (35%), and striking TUB-dysgyria (29%). All of these groups have the non-cortical malformations noted above, which are usually severe. Another important subgroup had mixed agyria-pachygyria without non-cortical malformations except mildly small cerebellum similar to the common *LIS1* pattern (14%). The latter patients all had mutations of a single codon: Arg402Cys or Arg402His.⁴

Mutations of *TUBB2B* were found in 10 of 811 (1.2%) patients, predominantly in those with tubulinopathy-associated dysgyria although one (LR00-059) had severe LCH with thin cortex similar to the most severe *TUBA1A* pattern. The Glu421Lys mutation was first reported in a family with polymicrogyria and congenital fibrosis of the extraocular muscles (CFEOM).²⁴ We observed the same mutation in a patient (LR03-060) with posterior predominant TUB-dysgyria, enlarged tectum and cerebellar vermis hypoplasia with no evidence for CFEOM. The mutation arose *de novo*, raising the possibility that the CFEOM phenotype may not be due solely to the *TUBB2B* mutation. Mutations in *TUBB* (previously designated *TUBB5*) and *TUBB3* were rare causes in our cohort (<1%), both associated with TUB-dysgyria with either nearly normal head size (n=3) or microcephaly (n=3). While each of the above LIS patterns with non-cortical malformations are recognizable as tubulinopathies, the most likely tubulin gene is usually difficult to predict.

Microtubule motor proteins

To date three genes encoding molecular motors have been associated with LIS: *DYNC1H1*, *KIF2A* and *KIF5C*.^{8,25-27} We found no mutations in either kinesin, although all reported patients presented with LIS or dysgyria.^{8,26,27}

Mutations of *DYNC1H1* were detected in 23 of 811 (3%) patients, making this the fifth most frequently mutated gene in our entire LIS cohort (but likely the fourth most common if we attribute our large number of *ARX* mutations to active ascertainment). We found 22 missense and one potential splice site mutations. Both parental samples were available for 13 patients, all confirmed to be *de novo*. Mutations were distributed throughout the entire gene with most comprising novel private mutations (n=14). Two previously reported mutations (Arg309His and Arg3344Gln) were recurrent in our cohort,^{8,9} and another was a different substitution at one of the same amino acids (Arg3344Trp). The mutations we found were located in tail as well as in motor domains, and were found in each of the six AAA domains as well as the microtubule-binding locus.

Following our initial reports of consistent differences in the gyral pattern between patients with *DCX* and *LIS1* mutations (anterior or posterior predominant, respectively),²⁸ every new LIS gene discovered has also had a consistent gradient. The motor proteins are the first group in which we have seen both posterior- and anterior-predominant gradients (Supplementary Figures 1 and 2). Most patients with mutations of *DYNC1H1* have a typical posterior-predominant gradient (n=16), but a few have had perisylvian-predominant

(posterior variant, n=3) or even anterior-predominant (n=5) LIS. We found no differences in mutations between patients with different LIS gradients.

Two unrelated patients with the same *KIF5C* mutation (Glu237Lys) had mild frontal LIS or dysgyria,^{8,26,27} while another with a different mutation (Arg268Ser) had posterior-predominant pachygyria.⁹ The two patients with *KIF2A* mutations had posterior-predominant pachygyria.⁸

Actins and actin associated MAPs (AAPs)

The critical role of the actin cytoskeleton became obvious when mutations of genes encoding for both non-muscle actins – *ACTB* and *ACTG1* – were linked to LIS,²⁹ and was reinforced with recent data showing that *DCX*, an atypical MAP that enhances microtubule polymerization, stabilizes the microtubule network and has several other functions, dynamically regulates formation of filamentous actin in developing neurons.²⁰ Integrations between the microtubule network and actin cytoskeleton are essential for critical cell functions such as cell division, neuronal migration, and axonal growth.³⁰ *DCX* binds directly to microtubules and, through interactions with actin-binding proteins such as sphinophilin, interacts with F-actin.^{19,31} Loss of *DCX* leads to re-distribution of actin from neuritis and growth cones to the cell body.²⁰

The role of *DCX* as a cross-linker of microtubules and the actin cytoskeleton is also reflected in *DCX*-associated phenotypes. The same anterior-predominant thick LIS seen with mutations of *ACTB* and *ACTG1* occurs with less severe mutations of *DCX*. Further, *ACTB* and *DCX* share the same striking histopathology. We previously described the neuropathological features in postmortem brain in both males and females with *DCX* mutations.³² At the cortical-white matter margin, the thick cortex transitions to multiple small nodules of subcortical heterotopia.³² Remarkably, the exact same pattern was observed in a girl in whom we subsequently detected a pathogenic mutation in *ACTB* (patient 1 in Forman is LP90-050 in Riviere^{29,32}). Severe especially early truncating mutations of *DCX* result in severe LIS with near complete agyria, morphologically indistinguishable from the severe phenotypes caused by *LIS1-YWHAE* deletion or certain *TUBA1A* mutations. We hypothesize that mild mutations of *DCX* have a primary impact on the actin cytoskeleton, while more severe mutations disrupt multiple functions, resulting in a more severe LIS phenotype.

DCX was the second most frequently mutated gene, accounting for 23% of patients with LIS including many with SBH. Our targeted sequencing study detected only three additional patients, none of whom had prior genetic testing. All three mutations were previously reported in the literature or listed in the ClinVar database. In males, *DCX* mutations cause diffuse or frontal-predominant LIS (near complete agyria or frontal pachygyria), and rarely frontal pachygyria with a transition to posterior SBH, or thin SBH.^{6,28} In females, mutations of *DCX* cause diffuse or thin, frontal-predominant SBH.

Mutations in *ACTB* and *ACTG1* each accounted for ~1% of patients in our LIS cohort. Both genes are associated with BWCF. Most patients with *ACTG1* mutations, and about 60% with *ACTB* mutations have mild frontal-predominant pachygyria, sometimes with transition

to posterior SBH, an appearance identical to less severe *DCX* mutations in males. We recently reported that *ACTG1* mutations can also cause frontal LIS with unspecific facial features not recognizable as BWCF. ³³

Complex MAPs

We classified *CKD5* separately because it has so many intracellular functions, ³⁴ and because we have data on only a single mutation, which was associated with diffuse LIS on selected images from a single patient. ¹² Based on the thick cortex, agenesis of the corpus callosum, large tectum, and severe CBLH, the imaging pattern resembles a tubulinopathy, suggesting disruption of multiple signaling pathways.

Reelin signaling

Homozygous or compound heterozygous mutations of the Reelin pathway genes *RELN* and *VLDLR* cause a unique pattern of malformations that consists of anterior-predominant “thin” LIS (Supplementary Discussion) with severe hippocampal and cerebellar hypoplasia. *RELN* mutations were a rare cause of LIS, accounting for only ~1% of patients (Table 1) including two children with compound heterozygous mutations in *RELN* – one missense mutation plus deletion of a single exon in one girl as well as two truncation mutations in another. At least two deletions of single exons in *RELN* are listed as rare CNVs in the control population (DGV, accessed January 2017), strongly suggesting that any molecular testing must be accompanied by copy number analysis. All children had anterior predominant thin LIS with diffuse severe cerebellar hypoplasia. However, one boy with a homozygous nonsense mutation (LP96-078, *RELN*:NM_173054.2:c.1249C>T:p.Gln417*) had a more severe phenotype than any previously reported, and died at home in the second decade (Supplementary Table 3).

Reelin (*RELN*) is an extracellular protein secreted by Cajal-Retzius cells in developing brain that controls neuronal migration and aggregation through multiple molecular mechanisms. ^{20,35} One of these involves regulation of the actin cytoskeleton via Cofilin activation. ^{20,35} This group and our actin and actin-associated MAP group share anterior predominant LIS patterns.

Forebrain transcription factors

The relatively high frequency of XLAG in our cohort most likely reflects very active recruitment for several years before and after gene discovery. The true frequency is likely to be closer to 1% or less. We provide additional genotype-phenotype analysis in the Supplementary Discussion.

Caspase-mediated apoptosis

We recently identified recessive mutations of *CRADD* in six individuals with thin LIS and normal cerebellum, and either true or relative megalencephaly. ¹¹ In contrast, the remaining 14 individuals with thin LIS had normal or small head size. We demonstrated that *CRADD*-associated LIS is caused by decreased neuronal apoptosis due to failure to activate caspase-2. ¹¹ Therefore, reduced apoptosis is a novel developmental mechanism for cortical malformations, although it accounts for less than 1% of LIS patients. However, mutations of

CRADD explained a third of patients with thin LIS and normal cerebellum, and confirmed clinical reports that thin undulating LIS with and without cerebellar anomalies often has autosomal recessive inheritance.

Diagnostic testing and genetic counseling

We integrated all of these data to develop a diagnostic testing strategy for medical professionals with no special expertise in malformations of cortical development or MRI interpretation (Figure 3 upper panel). This approach requires only the recognition of a cortical malformation and its classification as a type of lissencephaly. For specialists with substantial expertise, the diagnostic pipeline can be modified based on the specific pattern of LIS recognized (Figure 3 lower panel and legend). Our results demonstrate a diagnostic yield above 80% for most morphological LIS groups, with lower yields for frontal predominate thick pachygyria, dysgyria, partial posterior SBH and frontal thin LIS with normal cerebellum.

As may be expected with such a high yield from genetic testing, our experience has shown that negative testing of the known LIS-associated genes significantly reduces heterogeneity. Even with negative testing, the parents of children with thin (undulating) LIS should be counseled for possible autosomal recessive inheritance.¹ In contrast, autosomal recessive inheritance with thick LIS is possible, but very rare. Dysgyria with no molecular cause should be counseled for any inheritance patterns, as the available data is still insufficient to reliably predict the most common pattern of inheritance.

Summary

We provide a systematic analysis of the genetic basis of LIS-SBH using the largest cohort ever assembled. The total diagnostic yield from mutation analysis of 17 known LIS genes plus deletion 17p13.3 was 81%, with the four most frequent genes – *LIS1*, *DCX*, *TUBA1A* and *DYNC1H1* – accounting for 69%. We demonstrate a consistent phenotypic spectrum, defined primarily by brain imaging features especially by the anterior to posterior gradient, for each pathway-defined group. From our experience, recognition of one of these imaging patterns can support analysis of the many variants of unknown significance associated with genetic testing. Further, the genetic testing results (even when negative) and brain-imaging pattern combine to robustly predict both the most likely patterns of inheritance as well as the most likely clinical outcome, making them important for clinical management of patients. For example, recognition of SBH (especially in females), frontal predominant classic (thick) LIS and the temporal predominant XLAG pattern of LIS all predict X-linked inheritance (either *DCX* or *ARX* mutations). Similarly, recognition of thin undulating LIS with or without cerebellar hypoplasia predicts autosomal recessive inheritance. Most other patterns are associated with *de novo* heterozygous mutations, although parental gonadal mosaicism has now been recognized with several of these genes. Finally, most – but not all – SBH phenotypes are associated with mosaicism, including both true mosaic mutations (*LIS1*; also *DCX* in males more often than females) and functional mosaicism related to X inactivation in females (for *DCX*).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank the many patients and their families, as well as the physicians and genetic counselors who referred them, for their important contributions to this research over more than 30 years. Research reported in this publication was supported by the Deutsche Forschungsgemeinschaft (German Research Foundation, to N.D.D.), the National Institute of Neurological Disorders and Stroke (NINDS) of the National Institutes of Health under award numbers P01NS039404, R01NS050375, 1R01NS058721 and 1R01NS092772 (to W.B.D.), R01NS35515 (to M.E.R and W.B.D), and K08NS092898 (to G.M.M.), the National Institute of Child Health and Human Development under award number R01HD20619 (to D.H.L.), and by the EU Seventh Framework Program under the project DESIRE grant agreement N602531, and E-RareJTC2011 (to R.G.). Exome sequencing in this study was partly provided by the University of Washington Center for Mendelian Genomics (UW-CMG) and was funded by the National Human Genome Research Institute and the National Heart, Lung and Blood Institute grant U54HG006493 to Drs. Debbie Nickerson, Jay Shendure, and Michael Bamshad.

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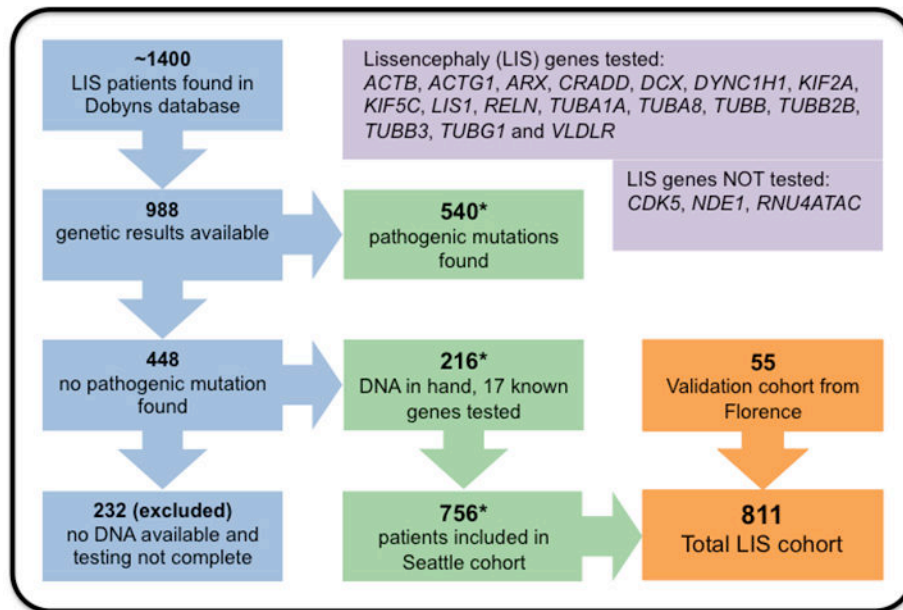


Figure 1. Flow chart describing subject selection for the primary study cohort (blue-green-orange panels), and lissencephaly genes tested and not tested (purple panels).

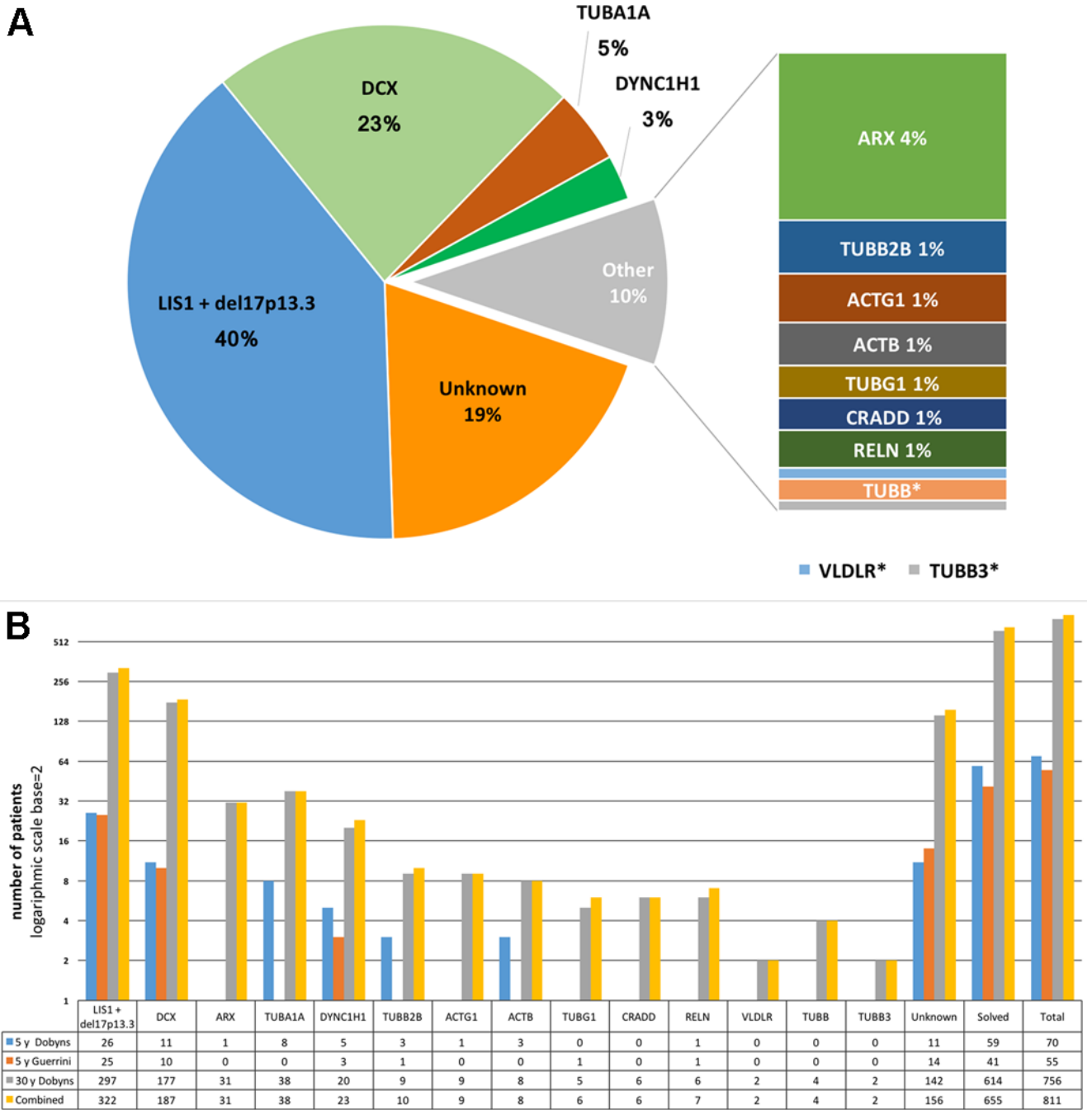


Figure 2. Number and frequency of mutations detected in lissencephaly (LIS) cohorts. The upper panel (A) shows the number and relative proportion (on a log base 2 scale) of mutations in our LIS cohorts: 5-year Dobyns cohort represents the subset of subjects with LIS recruited in Seattle between 2010 and 2015, 5-year Guerrini cohort represents an independent cohort ascertained at A. Meyer Children’s Hospital in Florence; 30-year Dobyns cohort includes all patients with LIS ascertained in the Ledbetter or Dobyns labs since 1982; the Combined cohort sums the 30-year Dobyns and 5-year Guerrini cohorts. The table below the graph

shows the exact number of patients carrying mutations in each gene. The lower panel (B) is a pie chart showing the diagnostic yield per gene in the Combined cohort. *Mutations in TUBB, TUBB3 and VLDLR each accounted for less than one percent of subjects.

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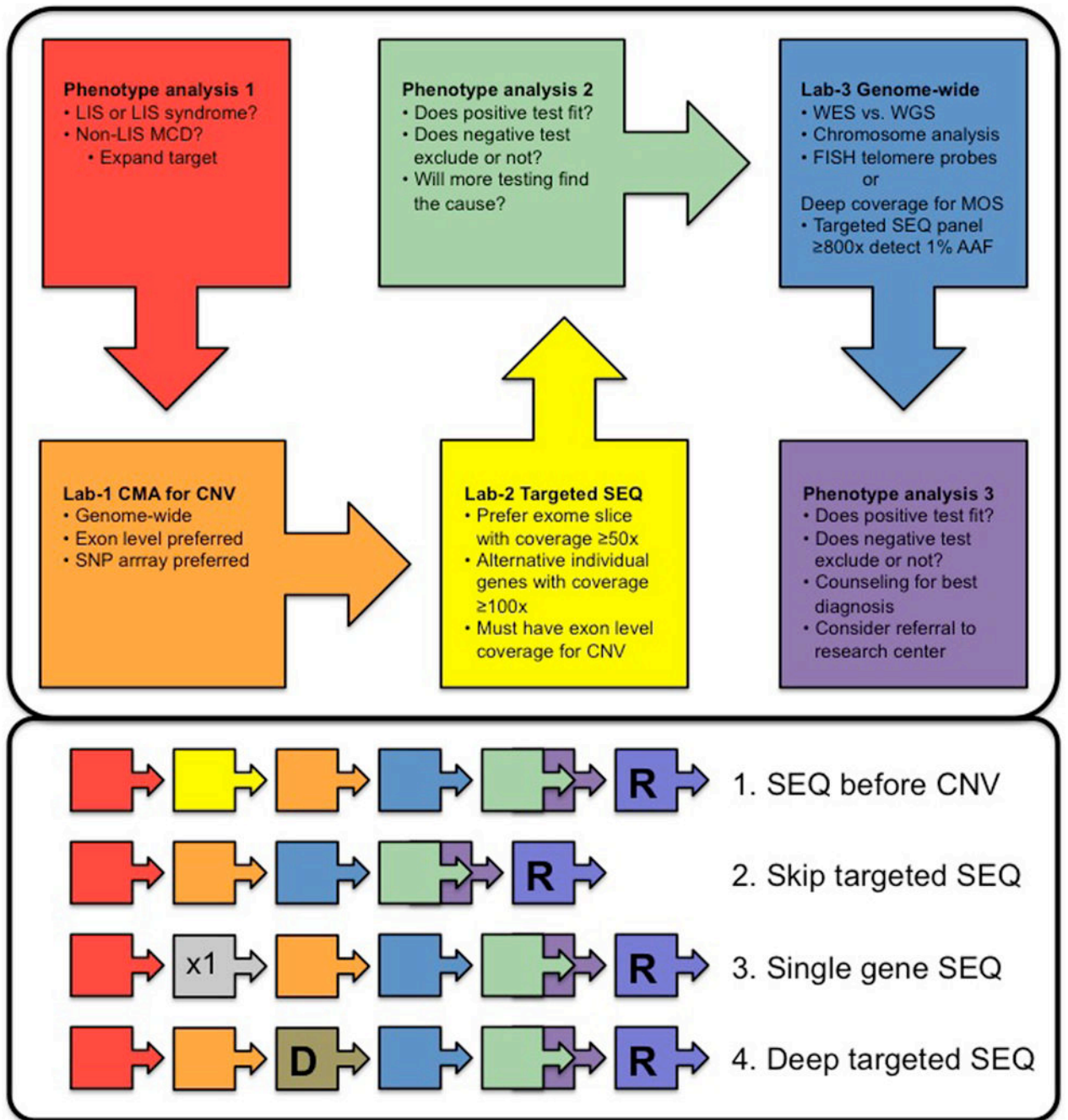


Figure 3.

Diagnostic algorithm. The upper panel shows a standard algorithm for genetic testing in patients with lissencephaly (LIS). Following initial clinical assessment (red box), a genome-wide chromosome microarray should be ordered to detect CNV (orange box), preferably an array with exon level coverage of most LIS genes. The next step is a targeted sequencing panel (yellow box). We recommend an exome slice approach, as this allows re-analysis for additional genes as new genes are reported or the phenotype of the child evolves to suggest tests for other disorders. The coverage of a standard exome is currently $\sim 50x$, sufficient to detect mosaicism with alternate allele fractions down to 20% with high reliability, and down

to 10% for some variants. The alternative approach of targeted individual gene sequencing at ~100x or greater will detect lower levels of mosaicism, although levels below 20% are rare with LIS-associated phenotypes. If not done in step 1, duplication-deletion analysis with exon level coverage of LIS genes should be done to detect small intragenic deletions and duplications missed by exome or individual gene sequencing (based on data to date, this is not needed for alpha and beta tubulin genes).

With these results in hand, phenotype re-review (green box) is useful to confirm that the phenotype matches any reported mutations, or if negative determine which type of LIS the phenotype best matches. When the child's condition and/or family's concerns support further testing, the next step is more complicated and involves either genome-wide testing such as whole exome sequencing, or deep targeted sequencing for low level mosaicism (blue box). A final phenotype review with all test results available is indicated for genotype-phenotype analysis and counseling (purple box). If the disorder appears to be rare or remains unsolved, referral to a research group for other approaches may be useful.

The lower panel shows several alternative approaches for testing that experts in LIS may choose to pursue. For example, sequencing may be performed before testing for CNV for tubulinopathies and disorders with autosomal recessive inheritance. For novel phenotypes, CNV testing could be followed directly by whole exome sequencing. Testing for X-linked LIS with abnormal genitalia (XLAG) could begin with single gene sequencing, while mild variants of LIS or subcortical band heterotopia might begin with deep targeted sequencing to be sure to detect mosaicism. Abbreviations: CNV, copy number variants; D, deep targeted sequencing; LIS, lissencephaly; MCD, malformation of cortical development; MOS, mosaicism; R, refer to research program; SEQ, sequencing.

Table 1

Genotype-phenotype correlation in the combined cohort of 811 LIS patients tested for known LIS-associated genes.

Protein network	CEN		TUB				MOTOR			AAP			Reelin		FORE	APOP	Solved	Unk	Total		
	LIS1-YWHAE	LIS1	TUBG1	TUBA1A	TUBB2B	TUBB	TUBB3	TUBA8	KIF5C	KIF2A	DYNC1H1	DCX (m)	DCX (f)	ACTB						ACTG1	RELN
Gene																					
Total #	75	247	6	38	10	4	2	0	0	23	73	114	8	9	7	2	31	6	655 (81%)	156 (19%)	811
Lissencephaly with cerebellar hypoplasia. Agyria diffuse (thick or thin cortex)				9	1														10 (67%)	5 (33%)	15
Classic thick lissencephaly																					
Agyria diffuse	75	17									25	1	1						119 (97%)	4 (3%)	123
Agyria-pachygyria p>a		130		5															135 (92%)	11 (8%)	146
Pachygyria p>a		48	6							8									62 (75%)	21 (25%)	83
Pachygyria p>a with non-cortical malformations		1		5	1					2									9 (90%)	1 (10%)	10
Agyria-pachygyria a>p										1	4								5 (83%)	1 (17%)	6
Pachygyria a>p											17	4	3	3					33 (72%)	13 (28%)	46
Pachygyria a>p with non-cortical malformations				1															1 (100%)	0	1
Pachygyria-band a>p (mixed)											9	4	4	6					23 (88%)	3 (12%)	26
Tubulinopathy-related dysgyria				11	8	2	1			7									29 (66%)	15 (34%)	44
Subcortical band heterotopia																					
SBH diffuse thick											5	57							62 (95%)	3 (5%)	65
SBH diffuse thin											2	8							10 (71%)	4 (29%)	14
SBH partial p>a		10																	10 (38%)	16 (62%)	26
SBH partial a>p											3	8							11 (85%)	2 (15%)	13
SBH unspecified												30							30 (81%)	7 (19%)	37
Thin undulating lissencephaly																					
Pachygyria a>p thin with cerebellar hypoplasia²															7	2			9 (75%)	3 (25%)	12
Pachygyria a>p thin with normal cerebellum																	6		6 (30%)	14 (70%)	20

Table 2

Classification of lissencephaly based on biological networks and brain imaging.

Protein networks	Genes	MRI - cortex: gradient and appearance					MRI - non-cortical brain malformations					
		P>A dysgyria	P>A thick LIS or SBH	Diffuse LIS or SBH	A>P thick LIS or SBH	A>P thin LIS	TL>P>A thin LIS	ACC	BG dysplasia	Large tectum	BS-CBLH	
Tubulins	TUBA1A	++	++	++				++	++	++		++
	TUBB2B	++	++	+				++	++	++		++
	TUBA8	++						+	+	+		
	TUBB	++						+	+	+		++
	TUBB3	++						+	+	+		++
Centrosome-expressed MAPs	LIS1-YWHAE*			++							±	
	LIS1		++								±	
	TUBG1		++									
	NDE1	++						++				++
Microtubule motor MAPs	DYNC1H1	++	++	-	+						+	+
	KIF5C		+	-	+	(+)					+	
	KIF2A		+	-		(+)					+	
	ACTB				++							
Actin and actin-associated MAPs	ACTG1				++					+		
	DCX			++	++							±
	CDK5**			+						+	?	+
Reelin signaling	RELN						++					++
	VLDLR						++					++
Forebrain	ARX									++	++	-
	CRADD										-	-

Number of “+” represents the frequency of the gradient within each group: ++ frequent, + less frequent, ± rare and mild involvement, (+) mimics phenotype.

ACC, agenesis of the corpus callosum; Actin-AAP, actin and actin associated proteins; AP, anterior predominant gradient indicates lissencephaly most severe in frontal poles and anterior frontal lobes but includes also lissencephaly extended through the posterior frontal lobes; BG, basal ganglia; BS-CBLH, brain stem and cerebellar hypoplasia; Forebrain, forebrain transcriptional regulator; LIS, lissencephaly; MAPs, microtubules associated proteins; PA, posterior predominant gradient indicates lissencephaly most severe in the parietal and occipital lobes including occipital poles; this group includes also perisylvian lissencephaly; TL, temporal predominant gradient indicates lissencephaly most severe in the temporal lobes and next most severe in parietal and occipital lobes; SBH, subcortical band heterotopia;

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* LIS1-YWHAE indicated deletion 17p13.3 (400kb Miller-Dickler critical region);

** provisionally classified as a complex MAP due functional links with *LIS1*, *NDE1*, *DYNC1H1*, *DCX*, and *TUBG1*, and imaging features (CBLH, ACC and severe tectal hyperplasia) that cross groups.