Characterization of Mutations in the Gene *doublecortin* in Patients with Double Cortex Syndrome

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Mutations in the X-linked gene *doublecortin*, which encodes a protein with no clear structural homologues, are found in pedigrees in which affected females show "double cortex" syndrome (DC; also known as subcortical band heterotopia or laminar heterotopia) and affected males show X-linked lissencephaly. Mutations in *doublecortin* also cause sporadic DC in females. To determine the incidence of *doublecortin* mutations in DC, we investigated a cohort of eight pedigrees and 47 sporadic patients with DC for mutations in the *doublecortin* open reading frame as assessed by single-stranded conformational polymorphism analysis. Mutations were identified in each of the eight DC pedigrees (100%), and in 18 of the 47 sporadic DC patients (38%). Identified mutations were of two types, protein truncation mutations and single amino acid substitution mutations. However, pedigrees with DC displayed almost exclusively single amino acid substitution mutations, suggesting that patients with these mutations may have less of a reproductive disadvantage versus those patients with protein truncation mutations. Single amino acid substitution mutations were tightly clustered in two regions of the open reading frame, suggesting that these two regions are critical for the function of the Doublecortin protein.

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Genetically inherited disorders of neuronal migration in humans represent important causes of epilepsy and mental retardation. Two clinically relevant disorders of neuronal migration include the "double cortex" syndrome (DC; also known as subcortical band heterotopia or laminar heterotopia) and X-linked lissencephaly. In these disorders, there is incomplete migration of neurons from the ventricular zone to the cortex during brain development. In DC, a subpopulation of neurons appear to arrest in the subcortical white matter, forming a second layer of gray matter.[1,2] In "classic" lissencephaly,[3,4] there is a failure of normal neuronal migration, leading to generalized disorganization of the cortex.

Most patients with DC are female, suggesting the action of an X-linked dominant disease. Pedigrees of

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X-linked lissencephaly,[5] or in which DC and lissencephaly were inherited in an X-linked fashion,[6] suggested that a single X-linked gene may cause both lissencephaly and DC (herein referred to as DC/XLIS). Presumably, affected males develop lissencephaly because all neurons express the mutant DC/XLIS allele. Females develop DC presumably secondary to X-inactivation, leading to two populations of migrating neurons. One population expresses the normal DC/XLIS allele and migrates correctly to establish the outer cortex. The other population of neurons expresses the mutant DC/XLIS allele and prematurely terminates migration to form the heterotopic band of neurons. Thus, mutations in the DC/XLIS gene are predicted to lead to DC only in females.[6,7]

Two studies demonstrated linkage of DC/XLIS to Xq21–24, with a pooled multipoint LOD score of approximately 4.2.[8,9] Subsequent positional cloning identified a novel gene, named *doublecortin* (*DCX* = gene; DCX = protein).[10,11] *DCX* mutations have been identified in several pedigrees displaying both DC and lissencephaly (DC/XLIS) and in sporadic patients with DC, confirming that females with heterozygous *DCX* mutations develop DC and that males with *DCX* hemizygous mutations develop lissencephaly. This study was undertaken to define the incidence of *DCX* mutations in patients and pedigrees with DC, to determine the sensitivity and specificity of molecular diagnosis in DC, and to determine if mutation identification can identify critical functional domains of the DCX protein.

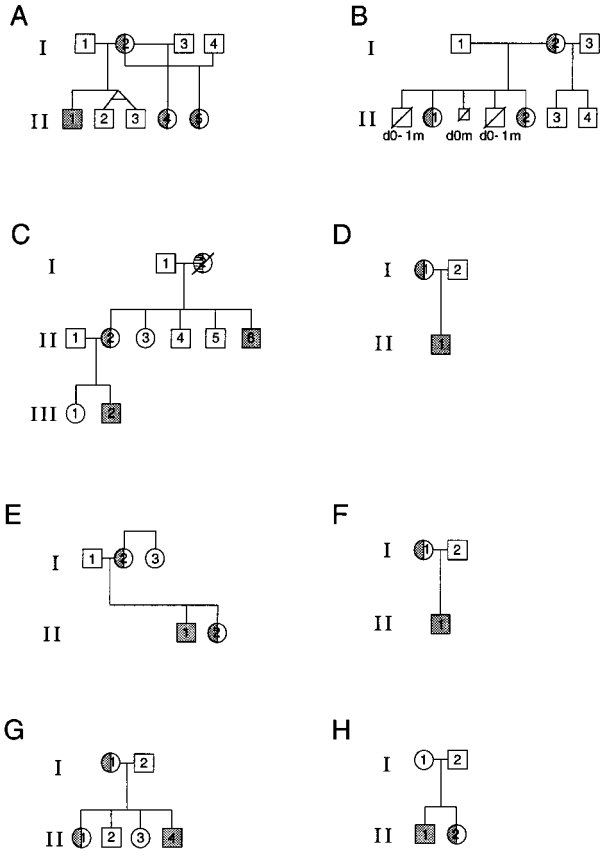

Fig 1. Pedigrees of patients enrolled in this study. Five of these pedigrees have been presented previously (Pedigrees A,[6,10,11] B,[10] C,[10] D,[10] and E[8]). Filled boxes = *males with lissencephaly;* half-filled circles = *females with double cortex syndrome. In Pedigree B, there were three mid-term to late-term/neonatal spontaneous abortions of males with death late in pregnancy* (d0m) *or 0–1 month of age* (d0-1m). *In Pedigree C, a grandmother* (half-hatched circle) *was known to display mental retardation and seizures, but a brain magnetic resonance imaging scan was not obtained, and so affection status is unknown. Pedigree H shows a clinically and radiographically normal mother with 2 affected offspring.*

Patients and Methods

Patients

Eight pedigrees (Fig 1) and 49 sporadic female patients with DC were evaluated for inclusion in this study. Two females with predominantly cortical pachygyria were excluded to minimize the chance of analyzing genetically heterogeneous diseases. All remaining patients showed (1) clinical symptoms including epilepsy and/or mental retardation; (2) a brain magnetic resonance imaging (MRI) scan interpreted as "subcortical band heterotopia" (double cortex) in females or classic lissencephaly in males[12]; and (3) nonprogressive disease that was not due to a previously recognized disorder. Representative brain MRIs from 4 females are presented in Figure 2. Patients were enrolled in accordance with clinical protocols approved by the institution review boards at Beth Israel Deaconess Medical Center, Children's Hospital, Boston, and the University of Minnesota Medical School, as well as several other participating medical centers. Patients recruited through the University of Minnesota were assigned separate identifying numbers, as follows: Pedigree A = LP95-080; Pedigree C = LP97-036; Pedigree E = LP95-136; Pedigree F = LP97-031; Pedigree G = LP96-031. The mutations for Pedigrees A, B, C, and D have been reported previously,[10] using identical identification letters, and Pedigree E has been reported for use in mapping (where it was referred to as Pedigree D[8]) and in a clinical descriptive article.[13] Pedigree A was also presented as Family 2 by des Portes and associates.[11] Of note, the mutation in Pedigree B had been reported incorrectly as a C-to-A point mutation at base pair 177 leading to an R59L amino acid substitution,[10] due to a typographical error during manuscript preparation. The mutations for sporadic Patients 2, 25, and 64 were also previously reported,[10] and they were referred to as Patients 2, 1, and 3, respectively.

Mutation Analysis

Genomic DNA was extracted from lymphocytes as previously described.[10] The polymerase chain reaction (PCR) primer sequences used to analyze the exons are listed in Table 1. Each exon was amplified by PCR and analyzed for single-strand conformational polymorphisms (SSCPs) on a nondenaturing polyacrylamide gel. To increase the sensitivity

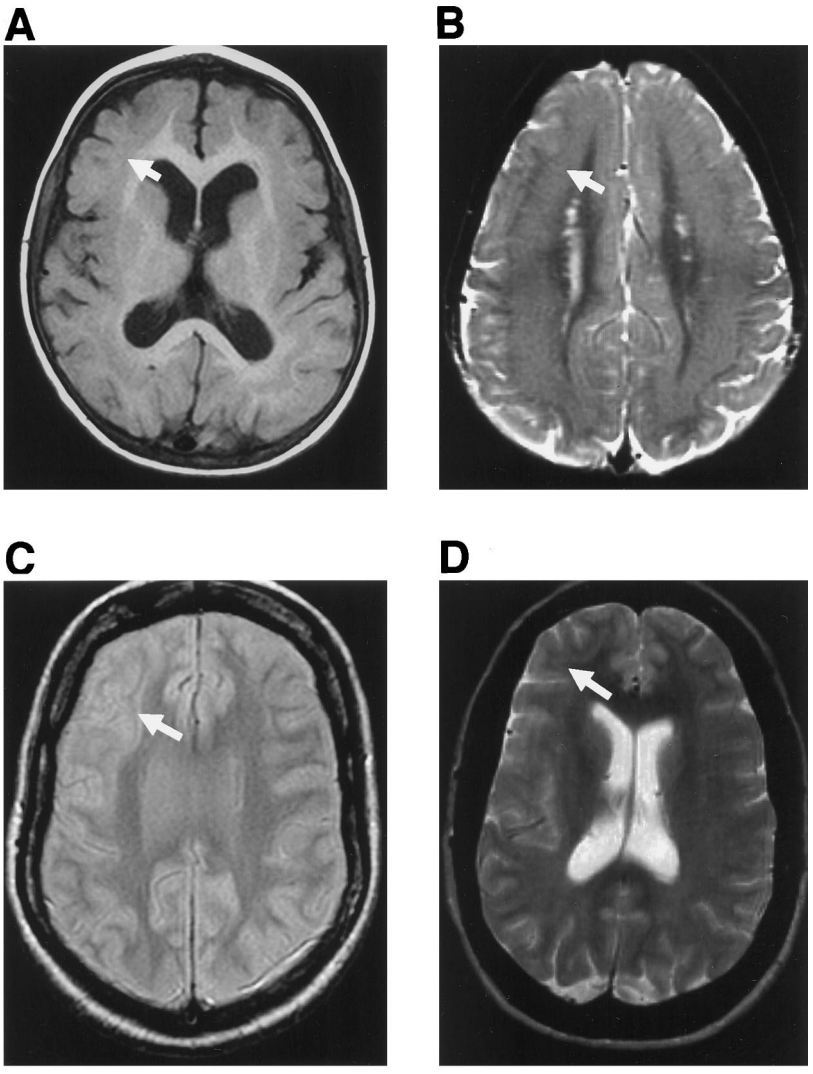

Fig 2. Brain magnetic resonance imaging scans from 4 representative patients included in this study; T1-weighted image from Patient 17 (A), T2-weighted image from Patient 64 (B), proton-density image from Patient C II-2 (C), T2-weighted image from Patient D I-1 (D). The band of heterotopic gray matter is indicated in each patient by an arrow. *Patients 17 and 64 display symmetrical bands that are present both frontally and posteriorly, and Patients C II-2 and D I-1 display symmetrical bands that are present predominantly frontally. The band in Patient 17 appears to fuse with the outer cortex in the frontal regions. Patient 64 displays a greater amount of overlying cortical pachygyria than either of the other 3 patients.*

of mutation detection, each exon was analyzed on two different SSCP gel matrix recipes, (1) a standard 4.5% nondenaturing polyacrylamide gel,[14] and (2) a mutation detection enhancement gel as recommended by the manufacturer (FMC BioProducts, Rockland, ME). Bands were visualized by silver staining according to the manufacturer's directions (Promega, Madison, WI). Polymorphic bands were excised from the gel, reamplified by using PCR, and sequenced in both directions to identify mutations as previously described.[10] The DNA from the parents of sporadic DC patients was also analyzed by SSCP to test whether the mutation had arisen de novo. Patient genomic DNA was also analyzed by Southern blot after restriction digestion with *Bam*HI and *Hin*dIII and probed with the full-length *DCX* cDNA, to test for gross rearrangements of the gene.

Statistical Analysis

Correlation of the data was assessed by statistical analysis, using a one-tailed Fisher exact test for two-by-two comparisons of the type of patient (sporadic or pedigree) versus the type of mutation (single amino acid substitution or protein truncation) and the probability of identifying a mutation.

Results

DCX mutations were identified in eight of eight pedigrees (100%) and in 18 of 47 sporadic patients (38%), and are tabulated in Tables 2 and 3. The probability of identifying a *DCX* mutation was significantly higher in pedigrees with DC/XLIS, compared with sporadic patients with DC ($p < 0.005$, one-tailed Fisher's exact test). Coding region mutations included 15 point mutations that produced predicted single amino acid substitutions in the DCX protein, four point mutations resulting in premature protein termination, and seven insertions or deletions of one or two base pairs resulting in frameshifts and premature protein termination. Each amino acid substitution mutation was nonconservative, from either a charged amino acid to an un-

Table 1. Polymerase Chain Reaction Primer Sequence Used to Amplify Each of the doublecortin *Exons*

doublecortin SSCP Primers	
Exon	Sequence
ORF exon 1af	CTTCACCCCCATCCCTTTCT
ORF exon 1ar	CAGCGTACACAATCCCCTTG
ORF exon 1bf	AGAACCTTGCAGGCACTGAG
ORF exon 1br	TAACCAATGATGCCACCTCC
ORF exon 2af	CACCTAATCACTTATTTCTTGCCTTAG
ORF exon 2ar	GCTCAAAAGAGTGGGCTGTC
ORF exon 2bf	AAGCTGGTTACCATCATCCG
ORF exon 2br	GAGTCCGTCAACAAGAAATGA
ORF exon 3f	GAGGTTCATTGTCACAGGACCA
ORF exon 3r	AAGGGGAGAGAACAATGGAGC
ORF exon 4f	TGTGTCCTTTTGCCCCAG
ORF exon 4r	TGTCCTCCATAAATGAAGTCAG
ORF exon 5f	TTCCTTTTCTCTTGCTTTGGG
ORF exon 5r	AACCTTCACCAAGCCATTCAG
ORF exon 6f	AGCAGACATTCCAGAGCTCAA
ORF exon 6r	GACTCTGAGCACTCTCCCCTC

Because the first two exons are more than 400 bases each, there are two pairs of primers to amplify each of these exons. The primers are complementary to intronic DNA surrounding each exon, to amplify the entire coding exon and the splice donor/acceptor sites.

SSCP = single-strand conformation polymorphism; ORF = open reading frame.

charged amino acid, or vice versa, presumably causing a significant change in DCX protein secondary structure. No neutral polymorphisms were detected in any patient or any of 30 control individuals. All *DCX* mutations were identified by SSCP screening; no polymorphisms were identified by Southern blot analysis. Figure 3 is a representative SSCP gel from Pedigree E and sporadic Patient 17.

We tested the hypothesis that sporadic DC patients tend to have more deleterious *DCX* mutations than familial DC patients, such as nonsense and frameshift mutations, as this is true for other disorders such as ataxia-telangiectasia.[15] Protein truncation mutations occurred in 10 of 16 sporadic patients, and amino acid substitution mutations were found in seven of eight pedigrees with DC/XLIS ($p < 0.05$, one-tailed Fisher's exact test). In fact, the only pedigree with a termination mutation (Pedigree H) did not show the mutation in the clinically unaffected mother, suggesting that the mutation was not inherited in a simple Mendelian fashion. This analysis suggests that DCX amino acid substitution mutations impart less of a reproductive disadvantage than protein truncation mutations.

The relationship of each mutation to the predicted DCX amino acid sequence is shown in Figure 4. This figure combines mutations from this study and from the two previous studies that identified mutations in DCX.[10,11] Protein truncation mutations appear to occur throughout the predicted DCX protein, but single amino acid substitution mutations may cluster in two regions. Amino acid substitutions are tightly centered near amino acid 75 (amino acid 47–125) and near amino acid 200 (amino acid 178–253); ie, these mutations occur approximately one-third and two-thirds

Table 2. Mutations in Sporadic Patients

Patient No.	*doublecortin* DNA Mutation	Predicted Doublecortin Protein Alteration
3	Point mutation G to A at bp 176	aa substitution R59H
5	Point mutation T to A at bp 749	aa substitution I250N
7	2-bp CT deletion at bp 684–685	Frameshift at aa 231; protein termination at aa 240
8	1-bp A deletion at bp 463	Frameshift at aa 155; protein termination at aa 156
9	Point mutation G to A at bp 706	aa substitution V236I
12	Point mutation T to C at bp 641	aa substitution I214T
13	Point mutation G to A at bp 705	Protein termination at aa 235
17	1-bp A deletion at bp 803	Frameshift at aa 267; protein termination at aa 273
20	Point mutation C to T at bp 665	aa substitution T222I
25	2-bp AG insertion at bp 36	Frameshift at aa 12; protein termination at aa 24
30	Point mutation C to T at bp 907	Protein termination at aa 303
35	1-bp A deletion at bp 31	Frameshift at aa 11; protein termination at aa 23
41	Point mutation C to G at bp 600	aa substitution N200K
42	2-bp TG deletion at bp 740–741	Frameshift at aa 247; protein termination at aa 254
44	Point mutation C to G at bp 608	aa substitution T203R
49	Point mutation A to G at bp 908	Protein termination at aa 303
64	2-bp CT deletion at bp 691–692	Frameshift at aa 231; protein termination at aa 240
127	Point mutation C to G at bp 572	Amino acid substitution P191R

Each *doublecortin* DNA mutation is numbered with reference to the ATG. The predicted Doublecortin protein alteration is numbered with reference to the amino acid (aa) residue number. Amino acid substitution mutations are referenced by the wild-type amino acid and position, followed by the mutant amino acid. For example R59H indicates that the wild-type amino acid R at position 59 is mutated to an H. One or two base-pair deletions or insertions result in a translational reading frameshift, followed shortly by a protein termination codon. Three patients (Patients 13, 30, and 49) have nonsense point mutations that result in a codon mutated to a stop codon, with resultant protein termination.

R = arginine; H = histidine; I = isoleucine; T = tyrosine; N = asparagine; K = lysine; P = proline; V = valine.

Table 3. Mutations in Pedigrees

Pedigree Reference	*doublecortin* DNA Mutation	Predicted Doublecortin Protein Alteration
A	Point mutation C to T at bp 574	aa substitution R192W
B	Point mutation G to T at bp 533	aa substitution R178L
C	Point mutation C to G at bp 608	aa substitution T203R
D	Point mutation A to C at bp 139	aa substitution S47R
E	Point mutation G to A at bp 758	aa substitution G253D
F	Point mutation G to C at bp 256	aa substitution D86H
G	Point mutation G to C at bp 299	aa substitution G100A
H	Point mutation C to T at bp 907	Protein termination at aa 303

The *doublecortin* DNA mutation is numbered with reference to the ATG. The predicted Doublecortin protein alteration is numbered with reference to the amino acid (aa) residue number. Amino acid substitution mutations are referenced by the wild-type amino acid and position, followed by the mutant amino acid. For example, R178L indicates that the wild-type amino acid R at position 178 is mutated to an L. One pedigree (H) has a nonsense point mutation that results in a codon mutated to a stop codon, with protein termination.

R = arginine; L = leucine; G = glycine; D = aspartic acid; W = tryptophan; S = serine; H = histidine; A = alanine.

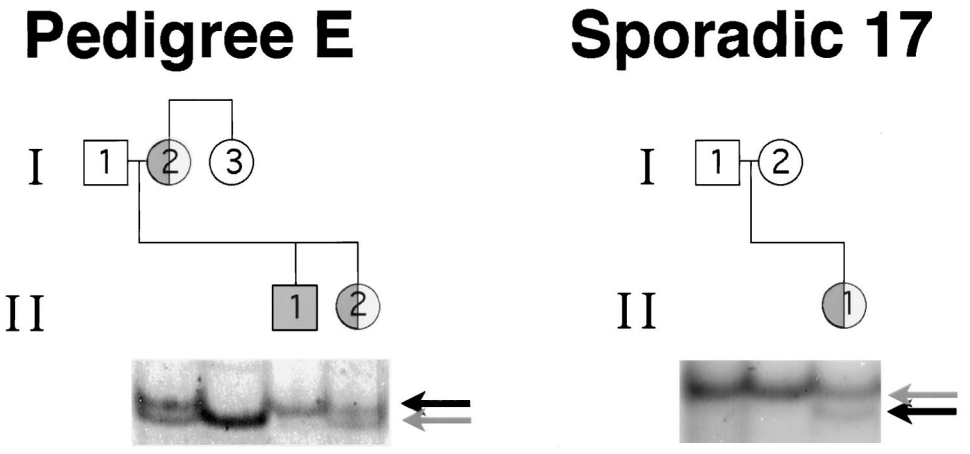

Fig 3. Representative mutation identification from Pedigree E and sporadic Patient 17, as detected by single-strand conformational polymorphism, using primer pair open reading frame exon 5. Filled box = *male with lissencephaly;* half-filled circles = *females with double cortex syndrome. Each unaffected individual displays a single normal band* (gray arrow), *the male with lissencephaly in Pedigree E (II-1) displays a single mutant band* (black arrow), *and each female with double cortex syndrome displays both a normal and a mutant band. The parents of Patient 17 display only the normal band, indicating that this mutation arose de novo.*

of the distance from the start codon. The first mutation cluster occurs near a tyrosine that closely matches a consensus phosphorylation site for c-Abl, a nonreceptor tyrosine kinase implicated in cell adhesion and migration.[16] However, none of the identified mutations lead to an amino acid substitution of the tyrosine predicted to be phosphorylated. The carboxy-terminus of the DCX protein, which encodes a serine/proline-rich region, is free of known amino acid substitution mutations.

Discussion

This study presents mutation analysis from a large cohort of patients with sporadic DC, familial DC, and familial DC plus lissencephaly (DC/XLIS). In DC patients meeting careful diagnostic criteria including brain MRI, SSCP identified mutations in 18 of 47 (38%) sporadic DC cases and eight of eight (100%) pedigrees. Identified mutations were of two types, single amino acid substitutions or protein truncation mutations. Amino acid substitution mutations were identified much more frequently in inherited DC, whereas protein truncation mutations were identified nearly exclusively in sporadic DC patients, suggesting that single amino acid substitution mutations may impart less of a reproductive disadvantage than protein truncation mutations. Single amino acid substitution mutations appear to cluster in two regions of the predicted amino acid sequence, suggesting that these two regions are critical for DCX function.

There are several possible explanations for why the proportion of patients with identifiable *DCX* mutations is higher in pedigrees with DC/XLIS than in sporadic DC patients. The first is that sporadic DC patients may have a higher proportion of non–coding region mutations. For some diseases, such as Unverricht-

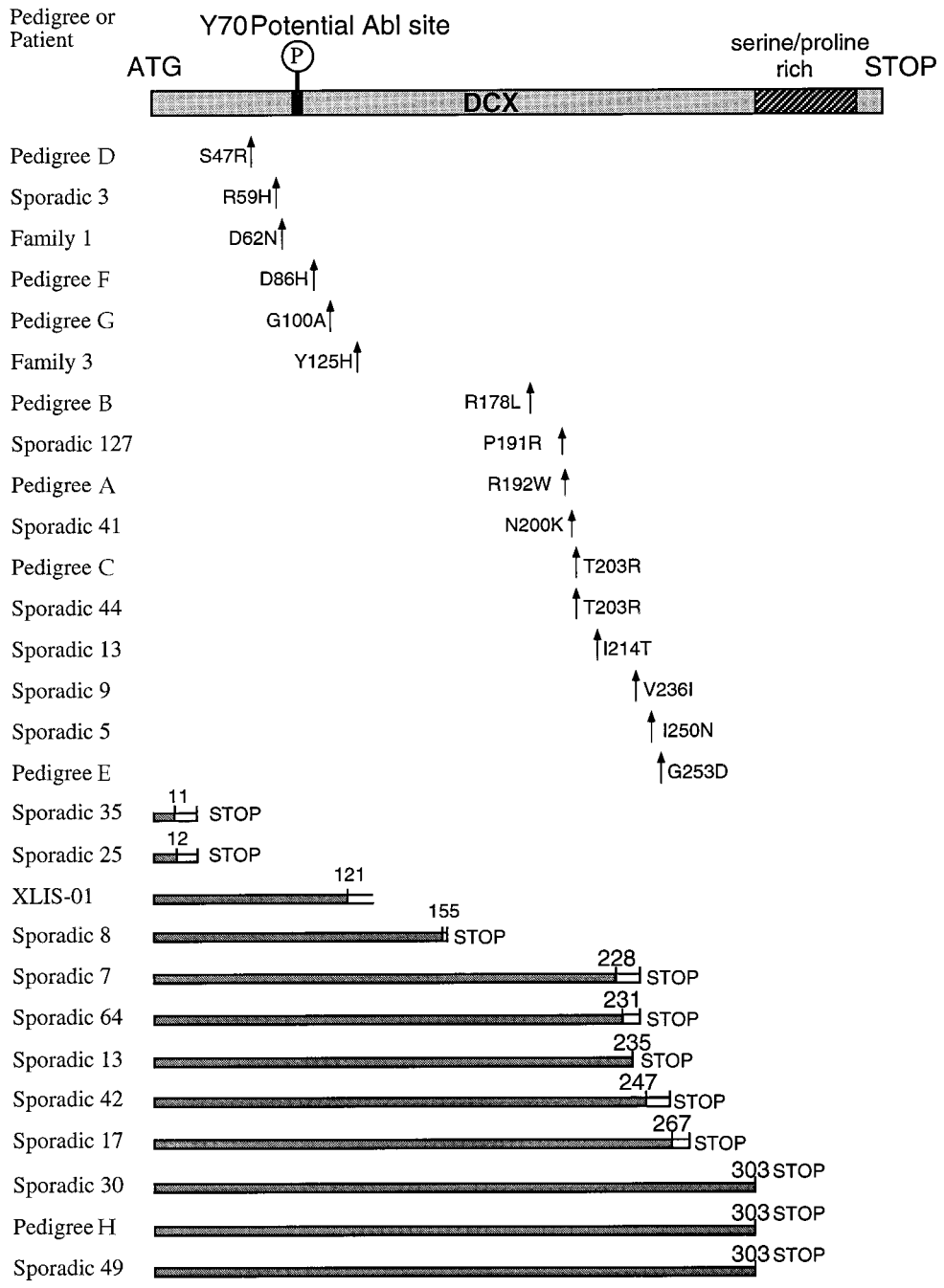

Fig 4. Schematic representation of mutations in each of the patients presented in this study and for the two previously published families (Families 1 and 3) from des Portes and associates.[11] Patient XLIS-01 is an affected sporadic female with a balanced chromosomal translocation previously reported.[10] Doublecortin protein (DCX) is represented as a solid bar extending from the start codon (ATG) to the stop codon at amino acid 330. Indicated are the potential Abl phosphorylation site at Y70, and the serine/proline-rich carboxy-terminus region. The top half of the figure tabulates the single amino acid substitutions as indicated by upward pointing arrows. *The bottom half of the figure tabulates the protein truncation mutations. The single amino acid substitutions are indicated by the original amino acid, the amino acid residue number, and the resultant amino acid. For example, Pedigree D has an S (serine) to R (arginine) substitution at amino acid position 47. The sites of the protein truncation mutations are indicated by shortened bars. The correct amino acid sequence is indicated by a* shaded bar, *the site of the mutation is indicated by the number, and nonsense amino acid sequence is indicated by an* open bar, *followed by a stop codon. Note that most pedigrees/families display single amino acid substitution mutations, and most sporadic patients display premature protein termination mutations. Also note that the single amino acid substitution mutations appear to cluster in two regions of the DCX protein, suggesting that these two regions are critical domains.*

Lundborg disease, the predominant mutation is outside of the coding region,[17] whereas coding region mutations are relatively rare. Perhaps analogously, there may be a fairly common mutation(s) in *DCX* that occurs outside of the coding region. If such a mutation produced a severe phenotype, it might be selected against in DC/XLIS pedigrees. In contrast, sporadic DC may be genetically heterogeneous. For example, mutations in an autosomal locus such as *LIS1* might also cause some cases of DC. Although this possibility cannot be excluded, it is unlikely, because each pedigree with DC/XLIS that has been tested[8,9] has linked to the Xq22.3–24 *DCX* region. The third possibility is that most sporadic DC patients do indeed have *DCX* mutations, but that SSCP screening is not a sufficiently sensitive means of mutation identification. Mutation screening by direct sequencing of each amplified exon from each affected patient may ultimately identify additional coding region mutations.

DCX mutations consisted of amino acid substitutions and protein truncation mutations, detected in roughly equal frequency (15 vs 11, respectively). However, amino acid substitution mutations were much more common in inherited DC, and no patients with protein truncation mutations have so far had children. Females with single amino acid substitutions in DCX may have less of a reproductive disadvantage compared with females with protein truncation. We hypothesize that amino acid substitution mutations may produce a milder neurological phenotype. A direct comparison of the clinical severity versus genotype is being pursued to help clarify this issue. In addition, these results are based on mutations identified in the minority of sporadic DC patients; further mutation identification in patients is needed to confirm this observation.

Single amino acid substitution mutations in DCX cluster in two regions, suggesting that these two regions may represent functionally important domains. When the single amino acid substitution mutations are combined from our data and from previously published data,[10,11] it appears that mutations tend to cluster around amino acids 47 to 125 and amino acids 178 to 253. BLAST sequence homology searches (Web address: http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-blast?Jform=0) of these two regions produce no matches to proteins of known enzymatic function. Perhaps these two regions function in novel protein–protein interactions. Study of the DCX protein may clarify the function of these regions; likewise, any model of DCX function must explain the striking clustering of these amino acid substitution mutations.

DCX now represents a second gene for human lissencephaly, in addition to *LIS1*.[18] The two genetic forms of lissencephaly show generally similar pathology of the cerebral cortex, but *LIS1* mutations are associated with more severe migrational abnormalities of the brainstem inferior olive.[13] Whereas at least 40% of *LIS1* mutations in patients with classic lissencephaly represent gross disruptions of the gene,[19] we found that most identifiable *DCX* mutations are point mutations or one or two base-pair insertions or deletions. Although these numbers may be somewhat skewed by ascertainment biases, the difference in mutational mechanisms in these two disorders is nonetheless striking. Further mutation analysis in patients with classic lissencephaly may better define the clinical genetics of these two disorders.

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