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ARTICLE

doublecortin is the major gene causing X-linked subcortical laminar heterotopia (SCLH)

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Subcortical laminar heterotopia (SCLH), or 'double cortex', is a cortical dysgenesis disorder associated with a defect in neuronal migration. Clinical manifestations are epilepsy and mental retardation. This disorder, which mainly affects females, can be inherited in a single pedigree with lissencephaly, a more severe disease which affects the male individuals. This clinical entity has been described as X-SCLH/LIS syndrome. Recently we have demonstrated that the *doublecortin* gene, which is localized on the X chromosome, is implicated in this disorder. We have now performed a systematic mutation analysis of *doublecortin* in 11 unrelated females with SCLH (one familial and 10 sporadic cases) and have identified mutations in 10/11 cases. The sequence differences include nonsense, splice site and missense mutations and these were found throughout the gene. These results provide strong evidence that loss of function of *doublecortin* is the major cause of SCLH. The absence of phenotype–genotype correlations suggests that X-inactivation patterns of neuronal precursor cells are likely to contribute to the variable clinical severity of this disorder in females.

INTRODUCTION

In the embryonic development of the mammalian brain, neurons migrate long distances to form the complex laminar structures of the cerebral cortex. The molecular mechanisms which guide the neuronal cells to their final destinations are not yet well understood; however, the study of disorders of neuronal migration can help to identify and characterize the genes and pathways involved. Such cortical malformation disorders, which are generally assessed by magnetic resonance imaging (MRI) or brain autopsy, are believed to be responsible for a significant proportion of the severe cases of epilepsy and cognitive impairment in children (1–3).

One such developmental disorder is subcortical laminar heterotopia (SCLH), or 'double cortex' syndrome (3,4). Interestingly, a skewed sex ratio has been observed in sporadic cases of SCLH: mainly female individuals are affected with very few male cases identified (5). In addition, several pedigrees have been identified in which affected males have lissencephaly or 'smooth brain' and affected females exhibit SCLH, which suggests that the same X-linked gene causes both diseases [X-SCLH/LIS (5,6)]. Lissencephaly is associated with profound mental retardation and epilepsy with many types of seizure, whereas heterozygous females with SCLH are generally less severely affected (7). Lissencephaly is defined by an absence (agyria) or a decrease (pachygyria) in surface convolutions of the cortex, which is disorganized and abnormally thick. Children with lissencephaly, in addition to severe mental retardation and epilepsy, often have microcephaly, marked hypotonia, no visual contact or speech and a shortened lifespan (8). SCLH presents with bilateral plates of heterotopic grey matter located between the cortex and the

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cerebral ventricles, below a thin band of white matter. The true cortex appears normal in lamination and is formed with correctly migrated neurons, whilst the heterotopic band consists of scattered neurons which have failed to migrate properly (3). Clinical manifestations are variable and seem to be related to the thickness of the heterotopic layer: intelligence can be normal or patients can exhibit varying degrees of mental retardation. Similarly, SCLH patients exhibit either occasional seizures or more severe forms of epilepsy and, in addition, there is often a delay in speech development and the ability to walk. Identification of the gene involved in this disorder is expected to lead to a more accurate classification of cortical dysgenesis conditions and to contribute to our understanding of the pathogenic mechanisms involved in epilepsy and mental retardation.

We and others have recently identified a novel CNS gene, *doublecortin*, and demonstrated its involvement in unrelated familial and sporadic cases of atypical SCLH (9,10). The predicted gene product shares significant homology over its whole length with the N-terminal segment of another protein, which is predicted to have a protein kinase domain at its C-terminus. Besides this, the function of *doublecortin* is unknown. It is highly expressed during brain development, mainly in fetal neurons, including precursor cells (9). It seems to be most highly expressed at mouse embryonic day 11, compared with later stages (10). Thus, the complete disorganization observed in lissencephaly and SCLH seems to reflect a failure of early events associated with neuronal dispersion.

In order to assess more fully the involvement of *doublecortin* in SCLH, we have now performed an extensive mutation analysis in a further 11 affected individuals. We have determined the intron–exon boundary sequences of *doublecortin* and have screened the splice sites and exonic sequences of all coding exons by a combination of denaturing gradient gel electrophoresis (DGGE) (11) and direct sequencing. In summary, nonsense, missense or splice site mutations were found in 10 of the 11 unrelated affected females. The high percentage of mutations detected confirms the major role played by this X-linked gene in SCLH.

RESULTS

Gene structure

The *doublecortin* gene has been shown to contain nine exons in total, although three exons at the 5′-end, 1A, 1B and 1C, are alternatively used. A putative start methionine in the first common exon (exon 2) has been identified, although exon 1C, if used, may extend the open reading frame at the 5′-end (9). The transcript size is 9.5 kb; however, a large proportion of this sequence is 3′-untranslated region (UTR). Intron–exon boundary sequences for all exons were determined by sequencing cosmid and phage genomic clones which encompass the gene. Sequences corresponding to the splice sites preceding and following each exon are presented in Table 1. These were found to adhere to consensus sequences with an invariant GT and AG in the splice donor and acceptor sites respectively.

Mutation analysis

In order to assess the degree of involvement of *doublecortin* in SCLH (which is observed mainly as female sporadic cases), we performed a mutation analysis on a collection of 11 independent patients with wide geographical origins. The clinical features of the patients are presented in Table 2. One family with SCLH (family 4) and 10 sporadic cases were examined in all potential coding regions of the gene. Genomic DNA from subjects was screened initially using DGGE. Primer pairs were designed either in intronic sequences to amplify whole exons (exons 1C and 4–6) or, alternatively, the exons were amplified in several overlapping pieces to generate products which had a suitable melting temperature (T_m) for the DGGE analysis (Table 3). Using this method, PCR fragments with an aberrant migration pattern, representing potential mutations, were identified and sequenced using direct automated sequencing. All coding sequences were examined in this way apart from PCR products corresponding to the 3.3 fragment of exon 3, which were directly sequenced and not analysed by DGGE.

To date we have identified 10 mutations in the 11 patients examined (Table 4 and Fig. 1). In case T.M. it was not possible to identify a mutation. Nine distinct point mutations were found, one of which was recurrent in two unrelated individuals: the C→T mutation at nucleotide position 971 in the cDNA sequence (DDBJ/EMBL/GenBank accession no. AJ003112) causes a predicted R186C missense mutation which segregates with the phenotype in family 4 and is present in case J.F. Three different nonsense mutations were observed, R39X (Fig. 2), Q235X and R303X, occuring respectively in exons 2, 3 and 5. In addition, one of the point mutations alters the splice consensus sequence at position –2 of exon 3 (AG→GG, case S.D.).

A total of five different missense mutations were observed confined to exons 3 and 4 (Fig. 1). One tyrosine residue at amino acid position 125 was affected by a T→G mutation (case O.D.), which is predicted to change the tyrosine to an aspartic acid residue. We previously found a mutation in this same tyrosine residue in an X-SCLH/LIS family (9). There are two predicted isoleucine to threonine changes at residues 214 and 250 which occur in cases J.A. and M.L. respectively. Case B.T. has a nucleotide alteration predicted to change Gly223 to a glutamic acid residue. Each of these mutations is predicted to change the Doublecortin protein significantly.

Table 1. Exon–intron boundary sequences

Exon	Exon/SD site	SA site/exon	Exon
1A	ACCAGCAGAAAACCG **gt**gagtggggctttt	gtcaaacgggaaaa**ag** CACAAGGCAAAGCCT	1B
1B	TTTTCCTTTGGAAAA **gt**gagtttgatgttc	ctttttttcccccc**ag** CTTATTTTTTATGAA	1C
1C	TAGGAGCCACTGTCA **gt**aagtctcaggatt	ttctcccacgctc**ag** GTCTCTGAGGTTCCA	2
2	ATGAACTGGAGGAAG **gt**aatttaaatagtg	tatttcttgcctt**ag** GGGAAAGCTATGTTC	3
3	CTGGATGGAAAACAG **gt**aggtacttttca	ctgctttctccata**g** GTAACTTGTCTCCAT	4
4	CTCTGGATGAAAATG **gt**aagcataaccact	gtccttttgcccc**ag** AATGCCGAGTCATGA	5
5	CTCCAGCTGACTCAG **gt**aacgaccaagacg	cttgctttgggct**ag** CAAACGGAACCTCCA	6
6	CTCCGGAAGCACAAG **gt**attatgtctctta	cttctcttcttat**ag** GACCTGTACCTGCCT	7

Table 2. Clinical data

No.	Family	Patient	Sex (M/F)	MRI: global aspect	Heterotopia thickness	Cortex	Age of walking (months)	Age of first speech (years)	Neurological examination	DQ/IQ	Epileptic syndrome
1a	1[a]	Moi.A	M	Agyria (CT)	None	Thick, agyric	No walking	No speech	Bedridden, little eye contact, spasticity of all 4 limbs	NA (<20)	Infantile spasms, symptomatic generalized epilepsy
1b	1	Mav.A	F	SCLH	++++	Pachygyric	NA	NA	Spastic gait on the left side	NA (severe MR)	Partial epilepsy, symptomatic generalized epilepsy
1c	1	Mag.A	F	SCLH (CT)	NA	NA	NA	NA	Normal	NA	Partial epilepsy
2a	2[a]	O.H.	M	Agyria	None	Thick, agyric	No walking	No speech	Bedridden, pyramidal syndrome, nystagmus, axial hypotonia	NA (<30)	Infantile spasms, symptomatic generalized epilepsy
2b	2	V.H.	F	SCLH	+ (frontal)	Normal	NA	NA	Normal	NA (low level)	Symptomatic generalized epilepsy
3a	3[a]	B.M.	M	Agyria–pachygyria	None	Thick, agyric (frontal) Pachygyric (posterior)	No walking	No speech	Bedridden, hypotonia	NA (<20)	Infantile spasms, symptomatic generalized epilepsy
3b	3	K.M.	F	SCLH	++	Moderately pachygyric (Frontal)	24	>3	Pyramidal syndrome, tremor, dyspraxia	NA (<60)	Symptomatic generalized epilepsy
3c	3	S.M.	F	SCLH	++	Normal	30	>3	Normal	66	Symptomatic generalized epilepsy
3d	3	I.M.	F	SCLH	±	Normal	30	NA	Normal	NA	Fits with fear, without loss of consciousness
4a	4	K.G.	F	SCLH	+++	Numerous gyri, malformation of insula	<18	NA	Normal	35	Partial epilepsy
4b	4	M.G.	F	SCLH	++++	Numerous gyri, malformation of insula	<18	NA	Axial hypotonia, limb hypertonia	NA	Partial epilepsy
4c	4	C.G.	F	SCLH	++	Normal	NA (normal)	NA	Normal	NA	Occasional seizures
5		S.D.	F	SCLH	+++	Pachygyric, numerous gyri, malformation of insula	16	2.5	Normal	GIQ = 52 (VIQ = 61, PIQ = 50)	Symptomatic generalized epilepsy, Lennox–Gastaut syndrome
6		O.D.	F	SCLH	++++	Pachygyric, numerous gyri, malformation of insula	36	4	Dysarthria, strabismus	37	Infantile spasms, symptomatic generalized epilepsy
7		J.F.	F	SCLH	+++	Numerous gyri, malformation of insula	No walking	No speech	Axial hypotonia, strabismus	NA (severe MR <50)	Partial epilepsy
8		S.K.	F	SCLH	++	Normal	18	3	Normal	NA	Symptomatic generalized epilepsy
9		M.L.	F	SCLH	+++	Numerous gyri, malformation of insula	24	2	Manual dyspraxia	74	Occasional seizures
10		T.T.	F	SCLH	+++	Pachygyric	24	NA	RAS	NA (severe MR)	Partial epilepsy
11[a]		J.M.	F	agyria–pachygyria	++++	Mixed agyria–pachygyria, malformation of insula	42	4.5	Ataxia, dyspraxia, visual and motor disturbance (left side), strabismus	30	Partial epilepsy (frontal?)
12		J.A.	F	SCLH	++	Pachygyric	18	>2	Dyspraxia	50	Infantile spasms, symptomatic generalized epilepsy; Lennox–Gastaut syndrome
13		T.M.	F	SCLH	+++	Pachygyric, numerous gyri, malformation of insula	16	2	Normal	50	Symptomatic generalized epilepsy
14		C.R.	F	SCLH	++	Pachygyric, numerous gyri, malformation of insula	18	>3	Normal	50	Symptomatic generalize epilepsy
15		B.T.	F	SCLH	+++	Numerous gyri	19	NA	Manual dyspraxia and gait disturbance	NA	Partial epilepsy

[a]Families or individuals studied previously (9).

++++, very thick; +++, thick; ++, moderately thick; +, slightly thick; ±, thin; NA; not available; DQ, developmental quotient; IQ, intelligence quotient; GIQ, global IQ; VIQ, verbal IQ; PIQ, performance IQ; MR, mental retardation.

Table 3. Conditions for PCR amplification and DGGE

Fragment	Sequence of primers	Length (bp)	Annealing temperature (°C)	Gradient (%)	Running time (h) at 160 V
Exon 1C	1C F, 5′-TTT TAT GAA TGT CGG ATA GCT GC-3′	433	55	20–70	11.5
	1C R, 5′-Pso-TA- GAG GAG AAG GGG AGA TTT TG-3′				
Exon 2.1	2.1 F, 5′-TCC CTT CTT TTT TCC CTT CTC C-3′	394	55	30–80	7
	2.1 R, 5′-Pso-TA- TGA GGC AGG TTG ATG TTG TC-3′				
Exon 2.2	2.2 F, 5′-ATC CAG GAA CAT GCG AGG CT3′	255	55	40–90	9
	2.2 R = 2.1 R				
Exon 2.3	2.3 F, 5′-TGA CCT GAC GCG ATCT CTG T-3′	148	55	30–80	6.5
	2.3 R, 5′-Pso-TA- ACC TCC CAC CAA CGG CCA CC-3′				
Exon 3.1	3.1 F, 5′-Pso-TA- CCT AAT CAC TTA TTT CTT GC-3′	183	55	30–80	6.5
	3.1 R, 5′-CTT GTT CTC CCT GGC CTG TG-3′				
Exon 3.2	3.2 F, 5′-TTG GCT AGC AGC AAC AGT GC-3′	176	55	30–80	6.5
	3.2 R, 5′-Pso-TA- AGT TTG ATG GCT TCT GTG AT-3′				
Exon 3.3	3.3 F = 3.2 F	260	50	[a]	[a]
	3.3 R, 5′-TAG AAA AAA CGG GAA AAG TAC TT-3′				
Exon 4	4 F, 5′-Pso-TA- TCA CAG GAC CAT CAT ATA CA-3′	219	55	5–55	5.5
	4 R, 5′-ACC CAT GGA AAT CCT AAA GG-3′				
Exon 5	5 F, 5′-Pso-TA- CCT CTA CTA AGC TGT CTG TG-3′	225	50	40–90	9
	5 R, 5′-TTG TCC TCC ATA AAT GAA GTC AG-3′				
Exon 6	6 F, 5′-Pso-TA- TTT ATC CCT TCC TTT TCT CT-3′	161	50	40–90	9
	6 R, 5′-AAG AGG TTT AGT AAG GTA TA-3′				
Exon 7	7 F, 5′-Pso-TA- AAC TTT GTC TCT TCT CTT CT-3′	119	55	30–80	6
	7 R, 5′-GGA TTT GTA CTC TGG ACT CTG A-3′				

[a]PCR products were sequenced directly.

Table 4. The cumulative spectrum of nonsense, missense and splice site mutations in the *doublecortin* gene

Mutation type	Position in cDNA[a]	Patient code	Mutation	Enzyme site change	Effect of mutation
Nonsense	Exon 2 (530)	Case S.K.	**C**GA→**T**GA	None	R (39) X
	Exon 3 (1118)	Case C.R.	**C**AG→**T**AG	None	Q (235) X
	Exon 5 (1322)	Case T.T.	**C**GA→**T**GA	None	R (303) X
Aberrant splicing	Exon 3 (–2 from 780)	Case S.D.	**A**G→**G**G acceptor site	*Sty*I (gain)	Splice exon 3, premature stop
	Exon 4 (+1 from 1223)	Case J.M.	**G**T→**A**T donor site	None	Splice exon 4[b], premature stop
Missense	Exon 2 (599)	Family 1	**G**AC→**A**AC	*Ava*II (loss)	D (62) N[b]
	Exon 3 (989)	Family 2	**C**GG→**T**GG	*Sty*I (gain)	R (192)W[b]
	Exon 3 (788)	Family 3	**T**AT→**C**AT	*Alu*I (loss)	Y (125) H[b]
	Exon 3 (788)	Case O.D.	**T**AT→**G**AT	*Alu*I (loss)	Y (125) D
	Exon 3 (971)	Family 4	**C**GC→**T**GC	*Pst*I (gain)	R (186) C
	Exon 3 (971)	Case J.F.	**C**GC→**T**GC	*Pst*I (gain)	R (186) C
	Exon 3 (1056)	Case J.A.	A**T**C→A**C**C	*Eco*RV (loss)	I (214) T
	Exon 3 (1083)	Case B.T.	G**G**G→G**A**G	*Msp*I (loss)	G (223) E
	Exon 4 (1164)	Case M.L.	A**TT**→A**CT**	None	I (250) T

[a]Nucleotide position in the cDNA sequence according to GenBank database entry AJ003112 starting from the 5′-end of exon 1C.
[b]Mutation reported in a previous study (9).

Other family members of the nine sporadic cases for which a mutation was identified were also tested for the presence of the mutation. In each case at least the mother of the affected individual was examined. PCR products amplified from genomic DNA were analysed by either restriction enzyme digestion, DGGE analysis or direct sequencing. In each case it was not possible to detect mutations in any of the other family members, which confirms the sporadic nature of the mutations.

A control set of >90 unrelated X chromosomes were tested for the presence of all but one of the splice site and missense mutations.

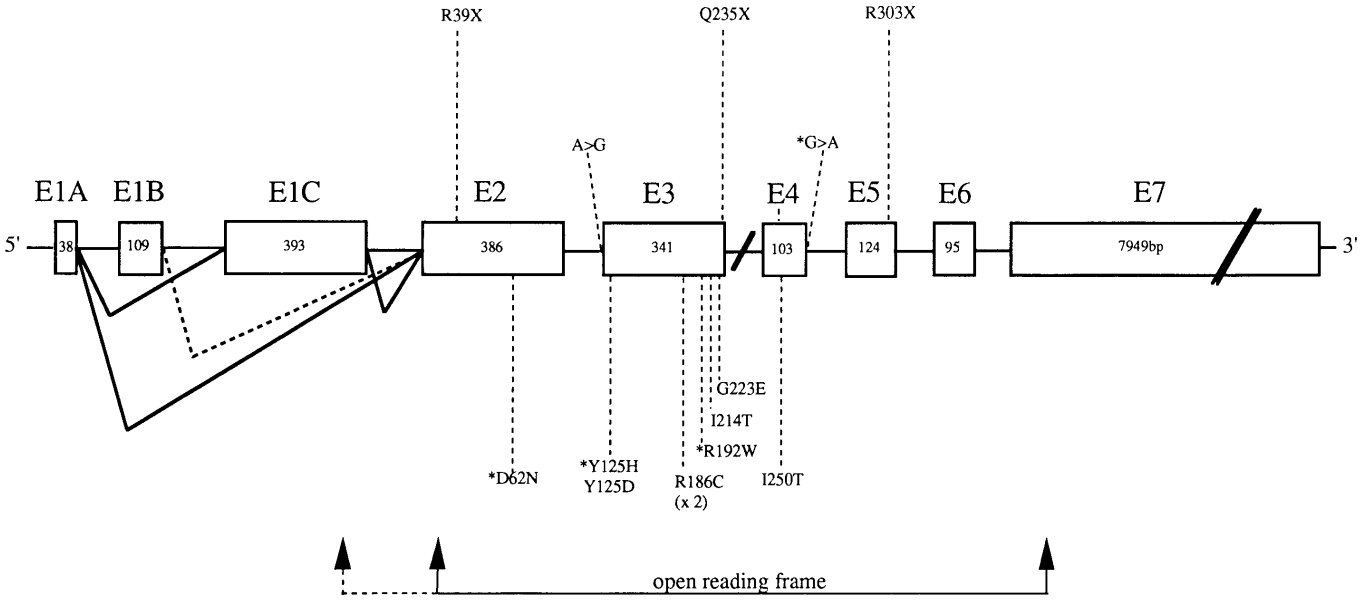

Figure 1. A schematic representation of the structure of the *doublecortin* gene. Exons are represented as rectangular boxes and their sizes in bp are indicated inside each box. Exons 1A, 1B and 1C each contain 5′-UTR sequences and have been found to be alternatively spliced to the remaining exons (exons 2–7). Nonsense and splice site mutations are represented above the exons and missense mutations below. Those mutations marked with an asterisk were identified in a previous study (9). The R186C mutation was identified in two unrelated individuals. 5′-UTR (exons 1A and 1B) and 3′-UTR sequences were not screened.

With the exception of the I250T missense mutation, all others led to the loss or gain of a restriction enzyme site (Table 4). None of the sequence differences was identified in the 90 chromosomes examined, which further verifies that these are significant disease-causing mutations and not coding polymorphisms.

Familial case

In the case of family 4 it is interesting to compare the clinical phenotype of the mother with those of her daughters (Table 2 and Fig. 3A–C). The mother has a relatively thin heterotopic band and suffers from only occasional seizures and thus appears to have a milder form of this disease. The two affected sisters were found to be heterozygous for the C→T mutation at nt 971 (R186C), which leads to the gain of a *Pst*I site. Interestingly, PCR amplification and digestion of the mother's DNA (case C.G.) was performed and the results suggest that she has only a partial gain of this *Pst*I site (Fig. 3D). Whereas the PCR products of the two affected heterozygous daughters show ~50% digested versus undigested products, the mother's DNA shows an unequal amount of the different amplified alleles (~20% have the *Pst*I site versus 80% without). PCR amplification and digestion were repeated three times for this family with reproducible results. As a further control the PCR products were also digested with another enzyme (*Alu*I), that has a restriction site 16 nt upstream from the mutated nucleotide. A complete digestion of the PCR product was observed for every family member with this enzyme (Fig. 3D). Sequencing of the PCR products reflected the results of the *Pst*I digestions, with both the normal and mutated nucleotide present in the mother's DNA sample in an uneven ratio (data not shown). In an attempt to quantify more precisely the percentage of alleles, hybridizations were performed to a Southern blot of *Pst*I-digested genomic DNA from members of this family. However, despite hybridizing with several different sized probes containing exon 3, it was not possible to obtain a unique hybridization signal, which may reflect the repetitive nature of the region and the presence of other *doublecortin*-like sequences in the genome. Although using the PCR technique it is not possible to quantify the percentage of the different alleles in this individual, our results suggest that she is mosaic for this mutation and this may explain her milder phenotype.

DISCUSSION

In a previous study we analysed three X-SCLH/LIS families and two sporadic SCLH cases by nested RT-PCR using lymphoblastoid cell lines (9). Three missense mutations co-segregating with the phenotype and a *de novo* splice site mutation were detected, demonstrating that defects in *doublecortin* were responsible for the X-SCLH/LIS syndrome. However, investigation of *doublecortin* transcripts carried out in one of the sporadic female cases did not reveal any abnormality. We suggested a poor efficiency of the RT-PCR-based approach in detecting heterozygous mutations due to inefficient amplification of the abnormal transcript and have hence implemented the DGGE method using genomic DNA for mutation screening in this new study. We now present the first systematic mutation analysis screen of all coding regions of *doublecortin*, examining 11 unrelated SCLH cases.

In addition to the four mutations we previously reported, we have now found mutations in 10 of the 11 new cases explored. Five patients had either nonsense point mutations or aberrant splicing leading to a premature stop codon and the five other cases, including one familial case, had point mutations leading to missense amino acid substitutions. In all of the SCLH individuals we have examined to date, no mutations have been identified in exons 1C, 6 and the coding part of exon 7. Two mutations were

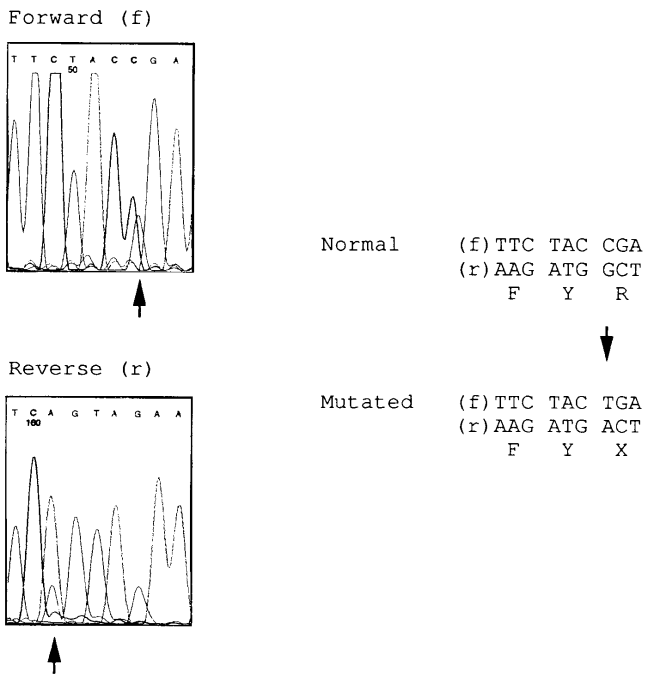

Figure 2. Sequence gel electropherograms showing a C→T point mutation (R39X) in a heterozygous female patient. The region of exon 2 sequence containing the mutation is shown on forward and reverse strands: a genomic DNA PCR product containing the exon 2.2 domain was purified and sequenced using primers 2.2 F and 2.2 R (Table 3). The PCR product contains both the normal and mutated alleles of the affected female patient, hence two bases are called at nucleotide position 530 (GenBank accession no. AJ003112). The C→T mutation (G→A on the reverse strand) changes codon 39 from an arginine to a stop codon.

observed in exon 2, eight in exon 3, two in exon 4 and one in exon 5. Hence, exon 3, which represents ~29% of the total coding sequence, contains 8/13 (62%) of the different mutations identified. Considering all the missense mutations, none was found in 90 control chromosomes examined, suggesting that they are not common coding polymorphisms. In addition, the missense mutations identified in the familial cases were all shown to co-segregate with the phenotype. Interestingly, only missense mutations have thus far been observed in male individuals with X-LIS, which may indicate that null mutations are embryonic lethal.

In one patient, case T.M., it has not been possible to identify a mutation. In this case mutations may be located in the unexplored 5′-untranslated region, the introns or the promotor sequences of the *doublecortin* gene. Alternatively, other loci may be involved and a gene which has strong homologies to *doublecortin* in its N-terminal region, KIAA0369, could be considered as a candidate gene (9,10). In addition, we looked for mutations in three males affected with SCLH. In these cases we did not detect a mutation (data not shown), suggesting that SCLH in males may represent a rare, distinct, perhaps autosomal, locus. Missense and frameshift mutations in *doublecortin* have also been found by other investigators in four out of nine pedigrees with X-SCLH/LIS and three additional female patients, although this mutation screen was incomplete (10).

The clinical severity of SCLH varies strikingly from asymptomatic clinical presentation with heterotopic bands assessed by MRI, to severe mental impairment with intractable epilepsy. The relative thickness of the heterotopic band seems to correlate with the phenotype, as patients with thicker bands have more severe mental retardation and seizures (12). Furthermore, an SCLH 'forme fruste', consisting of bilateral and symmetric bands with a regional distribution, has been described (13), which is associated with a milder phenotype. At first sight, a correlation between the clinical severity and mutation profiles might be suggested by our data. Indeed, four of the five nonsense and aberrant splice mutations leading to a premature stop codon occur in severely affected females with thick laminar heterotopia or pachygyria. However, this correlation remains unclear in other cases: (i) case S.K., who has a stop codon in exon 2, has a normal cortex and milder phenotype; (ii) in families 1 and 3, whereas the mothers and daughters share a constitutive missense mutation, the mothers have a thinner laminar heterotopia and a milder mental retardation than their daughters (14); in family 4 this also seems to be the case (Fig. 3) and we show that this is likely to be attributable to mosaicism; and (iii) missense mutations were observed in SCLH patients exhibiting variable severity and these mutations do not seem to involve functional domains such as putative PKC or CK2 phosphorylation sites, except R192W, which affects a predicted nuclear localization signal [(motif KPRK (15)]. Interestingly, eight of the 13 different mutations were detected within exon 3; however, correlations between the nature or the site of the mutation and the phenotype remain premature, since the critical functional domains of *doublecortin* are as yet unknown.

Furthermore, since SCLH is an X-linked condition, patterns of X chromosome inactivation in the brain could widely modulate the consequences of *doublecortin* mutations on cortical development. Indeed, assuming random patterns of inactivation in females with SCLH, functional Doublecortin should be absent only in cells which inactivate the X chromosome bearing the normal allele. This hypothesis is supported by histopathological data on SCLH brains (3) showing two populations of neurons, heterotopic cells lying in the white matter and cells that reach the normal six layered cortex. Strikingly, heterotopic cells are not rescued by neighbouring normal neurons, fitting in with a probable neuronal intracellular localization of *doublecortin* (9). Thus, since X chromosome inactivation occurs very early in development (16), different patterns of inactivation among neuronal precursors may explain phenotypic heterogeneity, especially laminar heterotopic band thickness and mental impairment. Taking into account this major phenomenon, correlations between mutations and clinical severity could be difficult to assess.

Our data show that *doublecortin* is the major gene involved in SCLH. Since we have identified mutations in atypical cases of SCLH, for example a 'forme fruste' (mother in family 4 with mosaicism causing occasional seizures) or severe forms leading to pachygyria and corpus callosum agenesia (case J.M.), it will be of interest to determine whether mutations in *doublecortin* contribute to other cortical dysgeneses or idiopathic forms of epilepsy. For instance, two previously reported pedigrees of X-linked dominant pachygyria associated with corpus callosum agenesia in males with decreased expressivity in female carriers (17–19) might be allelic disorders of X-SCLH/LIS. In addition, we might expect the involvement of *doublecortin* in some cortical

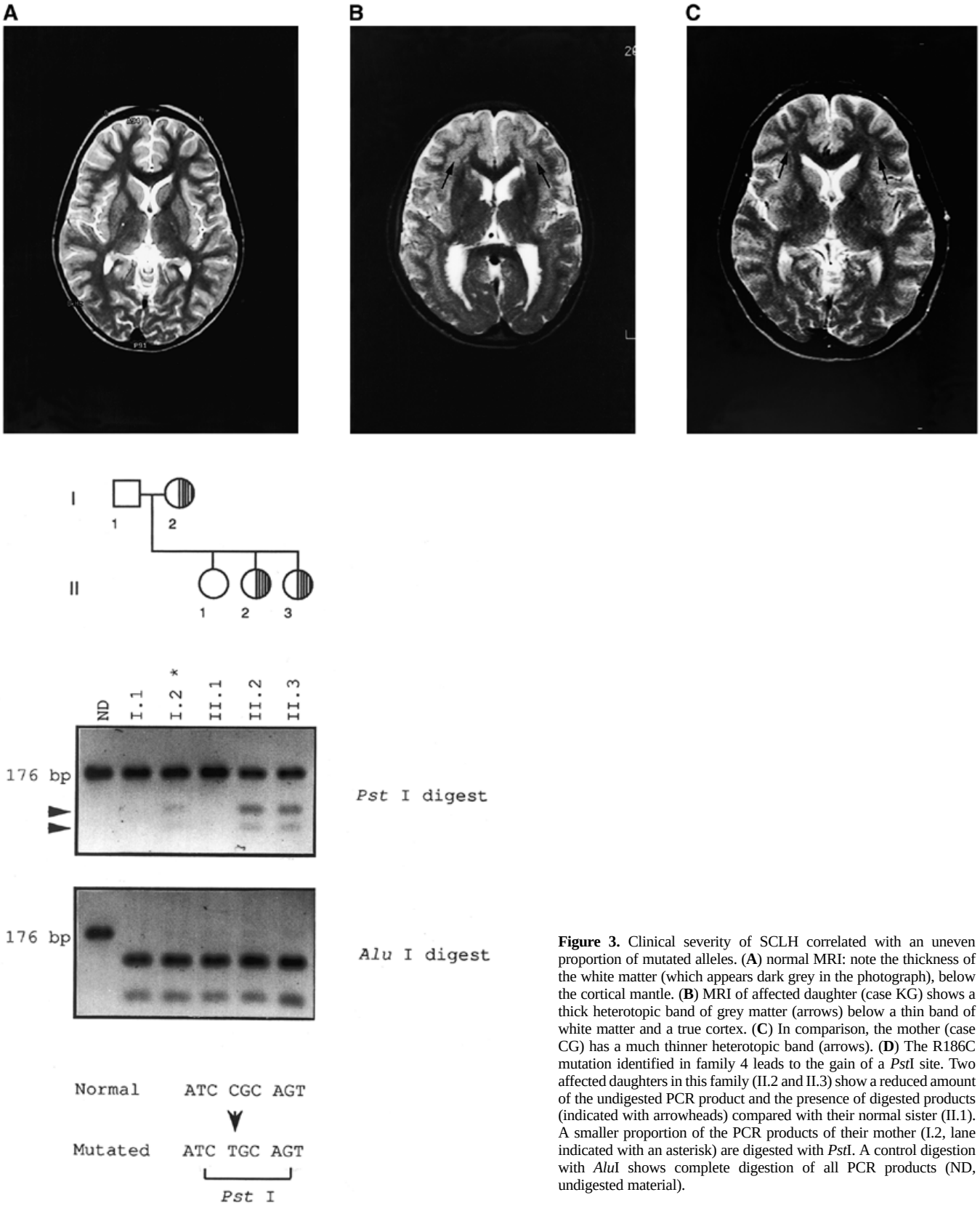

Figure 3. Clinical severity of SCLH correlated with an uneven proportion of mutated alleles. (**A**) normal MRI: note the thickness of the white matter (which appears dark grey in the photograph), below the cortical mantle. (**B**) MRI of affected daughter (case KG) shows a thick heterotopic band of grey matter (arrows) below a thin band of white matter and a true cortex. (**C**) In comparison, the mother (case CG) has a much thinner heterotopic band (arrows). (**D**) The R186C mutation identified in family 4 leads to the gain of a *Pst*I site. Two affected daughters in this family (II.2 and II.3) show a reduced amount of the undigested PCR product and the presence of digested products (indicated with arrowheads) compared with their normal sister (II.1). A smaller proportion of the PCR products of their mother (I.2, lane indicated with an asterisk) are digested with *Pst*I. A control digestion with *Alu*I shows complete digestion of all PCR products (ND, undigested material).

dysgeneses associated with isolated corpus callosum agenesia. The study of *doublecortin* and other related genes opens new avenues in understanding the pathophysiological mechanisms underlying cortical malformations and child epilepsy.

MATERIALS AND METHODS

Family material

The phenotype of each family member and routine MRI or CT scans were checked by the same paediatric neurologist. Each family member was included in the genetic study after obtaining informed written consent.

The patients originated from France, Italy, The Netherlands, India, Vietnam, Portugal, North Africa and the West Indies, and hence the dataset involved variable ethnic origins.

Gene structure

Cosmid and phage clones encompassing the *doublecortin* gene (9) were *Hin*dIII digested and subcloned into BluescriptSK (Stratagene). Subclones were end-sequenced and hybridized with gene-specific oligonucleotides to identify exon sequences and further sequenced with exonic oligonucleotides. Sequencing was performed using ABI dye terminator chemistry and an ABI sequencing machine and intron–exon boundary sequences were identified. 'Prediction of protein localization sites' (PSORT) was used to screen the amino acid sequence (15).

Mutation analysis

Lymphocyte DNA was obtained from Genethon, generated using standard methods. PCR reactions were performed using oligonucleotides specifically designed for use in the DGGE technique (Table 3). For each primer set one oligonucleotide contained a psoralen clamp at its 5′-end (20). In cases where more than one primer set was used to analyse an exon, primers were designed to amplify overlapping regions. PCRs were performed using a PTC-200 thermal cycler (MJ Research) at the annealing temperatures specified in Table 3, each for 40 cycles of 94°C × 40 s, annealing temperature × 30 s and 72°C × 40 s, with an initial denaturation and final extension of 4 and 5 min respectively. Taq and PCR buffer were obtained either from Eurogentec (Goldstar) or Gibco BRL and PCRs were performed at final concentrations of 0.25 mM dNTPs and 1.5 mM magnesium chloride. PCR products were analysed on agarose gels under standard conditions prior to the DGGE.

Heteroduplexes were allowed to form (94°C × 5 min, 55°C × 30 min) and PCR products were UV cross-linked (365 nm) for 15 min. PCR products were electrophoresed on polyacrylamide gels containing linear gradients of urea and formamide as specified in Table 3. The DGGE conditions for each type of PCR product were determined by assessing their melting profiles, using the Meltmap program (11). PCR products showing an abnormal migration pattern were directly sequenced on both strands.

Family members of individuals with identified mutations were checked where possible by restriction enzyme digestion (as detailed in Table 4). Restriction enzymes were used according to the manufacturers' specifications. In other cases PCR products of family members were checked by DGGE (cases S.K. and T.T.) or direct sequencing (case C.R.). Control individuals were mothers of Duchenne muscular dystrophy patients.

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