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Genetic alteration of the *DCX* gene in Japanese patients with subcortical laminar heterotopia or isolated lissencephaly sequence

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Abstract We examined mutations of the *doublecortin* (*DCX*) gene, which is responsible for X-linked subcortical laminar heterotopia (SCLH) and lissencephaly, in eight unrelated Japanese patients, four with SCLH and four with isolated lissencephaly sequence (ILS). Polymerase chain reaction (PCR) disclosed a deletion of part of the *DCX* gene in one male ILS patient. Single-strand conformational polymorphism analysis and subsequent sequence analysis were carried out in the remaining seven patients. One male ILS patient had a nonsense mutation in exon V, which would result in premature termination of the gene product. One female SCLH patient had a missense mutation in exon IV. Our results indicate that in the Japanese, as has been seen elsewhere, abnormality of the *DCX* gene is the common cause of SCLH and ILS.

Key words *DCX* gene · Subcortical laminar heterotopia · Isolated lissencephaly sequence

Introduction

Subcortical laminar heterotopia (SCLH) and lissencephaly are brain malformations caused by abnormal neuronal migration. SCLH consists of symmetrical and circumferential bands of gray matter located just beneath the cortex and separated from it by a thin band of white matter. The phenotype of affected females varies from nearly normal intelligence to severe mental retardation, but most patients have

seizures. Lissencephaly, on the other hand, produces profound mental retardation, seizures, and other neurological abnormalities. One gene responsible for lissencephaly was identified on chromosome 17p13 (Lo Nigro et al. 1997) and is called *LIS1* (Reiner et al. 1993). Only 38% of patients with isolated lissencephaly sequence (ILS) have visible cytogenetic or submicroscopic deletions of the *LIS1* gene (Dobyns et al. 1993), and only 10 of 25 ILS patients who had shown no gross deletions had mutations in the *LIS1* gene (Pilz et al. 1998). These findings may also reflect mutations in other genes responsible for ILS. SCLH and lissencephaly can be inherited alone or together in a single pedigree (Pinard et al. 1994). Among sporadic patients with SCLH, the sex ratio skewed toward females (Dobyns et al. 1996), suggesting the involvement of X-linked mutations. Linkage and physical mapping localized the responsible gene to Xq21.3-q24 (Ross et al. 1997). Recently, the *DCX* gene was isolated by two groups and shown to be responsible for SCLH and X-linked lissencephaly (des Portes et al. 1998a; Gleeson et al. 1998). They identified mutations in the *DCX* gene in 10 of 11 patients (des Portes et al. 1998b). *LIS1* and *DCX* mutations cause about 76% of ILS, but each of them is responsible for different patterns of malformation (Pilz et al. 1998). An extended study disclosed that the mouse *Dcx* gene encoded a putative Ca^{2+} -dependent signaling protein (Sossey-Alaoui et al. 1998).

In this study, we report that we have determined the intron-exon boundary sequences of the *DCX* gene and examined the *DCX* gene for alterations in eight Japanese patients, four with SCLH and four with ILS. The data presented here further support the conclusion that abnormalities of the *DCX* gene are responsible for SCLH and ILS.

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Patients and methods

Patients

DNA samples from eight Japanese patients known to have type 1 lissencephaly or SCLH were analyzed after the ob-

Table 1. Clinical diagnosis and sex distribution

Case no.	Clinical diagnosis	Sex
1	ILS	M
2	ILS	M
3	ILS	M
4	ILS	F
5	SCLH	M
6	SCLH	M
7	SCLH	F
8	SCLH	F

ILS, Isolated lissencephaly sequence; SCLH, subcortical laminar heterotopia; M, male; F, female

taining of informed consent, in accordance with established standards. Experienced neurologists diagnosed four of the patients with ILS and the other four with SCLH by their phenotype and brain magnetic resonance imaging (MRI). The clinical characterization of case 6 has been described elsewhere (Ono et al. 1997). None of the patients has a family history of mental retardation or seizure disorders. They were previously surveyed for genetic alteration in the *LIS1* gene located at 17p13.3, and none was found to have a mutation in the *LIS1* gene (Sakamoto et al. 1998). Samples were also obtained from the parents of case 7 and the parents of case 8. The clinical diagnosis and the sex of the subjects are shown in Table 1. None of the patients had any obvious cytogenetic abnormalities.

Genomic sequencing

YACs 749f7 and 737h4 (CEPH Mega YAC library), which included the entire genomic region of the *DCX* gene, were used for determining the intron sequences. Polymerase chain reaction (PCR) experiments were performed with a Gene Amp PCR system 2400 (Perkin-Elmer Cetus, Norwalk, CT, USA). To isolate genomic fragments corresponding to both splice acceptor and donor sites of all exons, inverse PCR was employed (Triglia et al. 1988). YAC DNA was completely digested with *MspI*, *TaqI*, *RsaI*, *AluI*, or *Sau3AI*, and self-ligated in a diluted condition. PCR was performed with oppositely directed primers located in *DCX* cDNA and with the self-ligated DNA as template.

Direct sequencing of the inverse PCR products was performed using the dideoxy chain-termination method (ABI PRISM DNA sequencing kit; Applied Biosystems, Foster City, CA, USA). Reactions were analyzed with an ABI 373S autosequencer according to the manufacturer's protocol (Applied Biosystems).

Mutation analysis

We performed single-strand conformational polymorphism (SSCP) analyses according to standard methods; in brief, each sample was electrophoresed at 250V across a precast 5% polyacrylamide Tris-borate electrophoresis buffer (TBE) gel in a room maintained at 4°C. After the gels were stained in a 1× solution of SYBR Green II (FMC

BioProducts, Rockland, ME, USA) for 5 min, bands were visualized and analyzed by means of an FMBIO II Multi-View fluorescent image analyzer (Takara, Tokyo, Japan).

Direct sequencing of PCR products from genomic DNA was performed on both strands by the dideoxy chain-termination method (ABI PRISM DNA sequencing kit; Applied Biosystems). Reactions were analyzed with the ABI 373S autosequencer according to the manufacturer's protocol (Applied Biosystems).

Fluorescence in situ hybridization (FISH)

A human genomic DNA cosmid library was constructed by a method described previously (Tokino et al. 1991). Screening was performed with α -³²P-dCTP labeled *DCX* cDNA as a probe.

Metaphase chromosomes of the patients were obtained from lymphoblastoid cell cultures. Dual-color FISH was carried out on the slides as previously described (Inazawa et al. 1994). The test probes were labeled with digoxigenin-11-dUTP by nick-translation; a biotin-labeled chromosome X alpha-satellite probe served as the control (Oncor Gaithersburg, MD, USA). The test probe and the control were detected simultaneously with avidin-fluorescein isothiocyanate (FITC) and antidigoxigenin-rhodamine (Boehringer-Mannheim, Berlin, Germany), after which the chromosomes were counterstained with 4,6-diamino-2-phenylindole (DAPI) (Sigma, St. Louis, MO, USA) in an anti-fade solution. Signals were examined on a Nikon Optiphot fluorescent microscope equipped with a charge-coupled device (CCD) camera (Nikon, Tokyo, Japan) and superimposed by means of Mac-Probe Version 2.5 software (Perceptive Scientific Instruments League city, TX, USA).

Results

In order to survey the patients with lissencephaly or SCLH for subtle genetic alterations in the *DCX* gene, we determined the genomic sequences surrounding the exon-intron boundaries at both donor and acceptor sites of the gene. We designed PCR primers for exon amplification so as to yield products including exon-intron junctions and comprising appropriate sizes for SSCP (Table 2). Inverse PCR, using the YAC as a template, successfully yielded the required PCR products including the junction sequences.

We performed SSCP analysis on the four SCLH and four ILS patients. In one male ILS patient (case 3), no PCR products were obtained between exons IV and VII in the *DCX* gene (Fig. 1). Southern analysis, using PCR products of these exons as probes, showed no corresponding bands in this patient (data not shown). These results suggested that this patient had a deletion in this region of the *DCX* gene. Three patients showed aberrant electrophoretic patterns when exons IV and V were screened. Direct sequencing of the PCR products from the genomic DNA of these patients revealed that one male ILS patient (case 2) had a nonsense

Table 2. Oligonucleotide primers used for exon amplification

Sense primers		Antisense primers		Product size (bp)
E1s	AGACTGCTTCCTAAGCTGGAGA	E1a	GGGAGTAAGAGATAGAGAGGGA	254
E2s	AGCAGCAGATTGCAGATCTGGA	E2a1	CAAGGCGTCAAAGCTGCGAAAA	388
E2s2	TTGTGTACGCTGTGTCCTCTGA	E2a2	TAACCAATGATGCCACCTCCCA	214
E3s1	GTTCTACTCCAGTGTCAAGTGTG	E3a1	CCACTGCGGATGATGGTAACCA	293
E3s2	CCAGGGAGAACAAAGGACTTTGT	E3a2	GAGGAAGAGTCCGTCAACAAGA	294
E4s	GAGGTTTCATTGTACAGGACCA	E4a	CCTAAAGGATAGAAGGGGAGAG	217
E5s	CCAGAGGCTGATAACATGCTGA	E5a	AAGTCAGCGTGCACAGTTAGGA	242
E6s	AGGGGAAGGATAACTTGCTCCT	E6a	GCTGTTGAGTTAGAATGGAAGAG	260
E7s	GCAGACATTCCAGAGCTCAAGA	E7a	ATAGCCCTGTTGGACACTTGAG	157

Figure 1

Fig. 1. Polymerase chain reaction (PCR) results for exon III 2 and exon IV of the *DCX* gene in eight patients and one normal control. Numbers on the left represent size markers of products. In one male ILS patient (case 3), no PCR products were obtained between exons IV and VII in the *DCX* gene

Figure 2a **Figure 2b**

Fig. 2a,b. Sequence analysis of PCR products from genomic DNA. Arrowheads indicate substituted nucleotides. **a** Case 2. A C-to-T change at nucleotide 907 was identified in exon V. This substitution causes codon 303 to change from CGA (arginine) to termination codon TGA. **b** Case 7. A C-to-T change at nucleotide 752 was identified in exon IV. This substitution causes codon 251 to change from GCC (alanine) to GTC (valine)

mutation (907 C < T) in exon V, which would lead to a premature termination of translation (Fig. 2a). One female SCLH patient (case 7) had a sequence variation (752 C < T) in exon IV (Fig. 2b), which is predicted to change the alanine residue at amino acid 251 to valine. We did not detect the same sequence variation in either of her parents, indicating that this sequence variation was *de novo*. Also, we did not detect this mutation in 100 normal control individuals, suggesting that it was not a polymorphism but a disease-causing missense mutation. Case 8 had a sequence variation near the branch point of exon V (809-44 G < A), but the sequence difference was also identified in the X chromosomes from normal control individuals examined, suggesting that this sequence variation was a polymorphism (data not shown).

In the one female ILS patient of the remaining two ILS patients, and the two female SCLH patients of the remaining three SCLH patients, we planned FISH analyses to identify submicroscopic heterozygous deletions, probed with two genomic cosmid clones of the *DCX* gene, one of which includes exons I–II, the other of which includes exons IV–V of the *DCX* gene. FISH analyses were carried out, but we found no submicroscopic deletions in the *DCX* gene.

Discussion

In this study, we analyzed the *DCX* gene for abnormalities in eight Japanese patients with either SCLH or ILS. We found alterations in one of four SCLH patients and two of four ILS patients.

Among the mutations found in the ILS patients, one was a deletion from exon IV to exon VII of the *DCX* gene and the other was a nonsense mutation in exon V (907 C < T, R303X); both mutations could be expected to lead to premature termination of translation and a truncated product lacking the C-terminus of *doublecortin*. Therefore, the loss of only the last 58 of 360 amino acids results in ILS. *Doublecortin* has potential mitogen-activated protein (MAP) kinase family phosphorylation sites at the C-terminus (Gleeson et al. 1998), which is therefore predicted to be important for regulation of its activity or protein stability. The nonsense mutation (907 C < T, R303X) is the same as that reported in two male patients with ILS (Pilz et al. 1998) and one female patient with SCLH (des Portes et al. 1998b).

Additionally, we found a missense mutation in exon IV in one SCLH patient. This mutation changes the amino acid at position 251 from alanine (GCC) to valine (GTC). The unaffected parents did not carry the sequence variation identified in their affected daughter, suggesting that this sequence variation is de novo. Both alanine and valine have an aliphatic sidechain and both are hydrophobic. Therefore, this amino acid change will not have much influence on the conformation of *doublecortin*. As expected, the patient has nearly normal intelligence, but still has seizures.

Our data lend support to the conclusion that, in Japan, as elsewhere, the *DCX* gene is often involved in the etiology of SCLH or ILS.

We failed to find any alterations in three SCLH patients and two ILS patients. SSCP analysis may have caused us to overlook some of the mutations in the region examined. We also cannot exclude the possibility that in these patients, subtle genetic alterations may be present within the promoter region or introns. *Filamin1* is known to be mutated in paraventricular heterotopia (PH; another disorder consisting of brain malformation caused by abnormal neuronal migration) (Fox et al. 1998). But we can exclude the possibility that *filamin1* is mutated in those patients who have no alteration in *DCX* gene, because their phenotypes are very different from that of PH, which consists of nodules lining the ventricular surface.

To summarize, we found *DCX* mutations in two male patients with ILS and one female patient with SCLH, findings that agree with the X-linked transmission of *DCX* mutations. Other patients in whom we could not detect a *DCX* mutation, particularly female patients with ILS and male patients with SCLH, also had no mutation in the *LIS1* gene (Sakamoto et al. 1998). These findings may also suggest that there are mutations in other autosomal genes responsible for SCLH or ILS.

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