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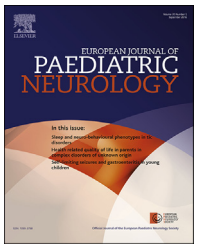

Case Study

A novel *DCX* missense mutation in a family with X-linked lissencephaly and subcortical band heterotopia syndrome inherited from a low-level somatic mosaic mother: Genetic and functional studies

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ABSTRACT

Purpose: To study the genetics and functional alteration of a family with X-linked lissencephaly and subcortical band heterotopia.
Methods: Five affected patients (one male with lissencephaly, four female with subcortical band heterotopia) and their relatives were studied. Sanger sequencing of *DCX* gene, allele specific PCR and molecular inversion probe technique were performed. Mutant and wild type of the gene products, namely doublecortin, were expressed in cells followed by immunostaining to explore the localization of doublecortin and microtubules in cells. *In vitro* microtubule-binding protein spin-down assay was performed to quantify the binding ability of doublecortin to microtubules.
Key findings: We identified a novel *DCX* mutation c.785A > G, p.Asp262Gly that segregated with the affected members of the family. Allele specific PCR and molecular inversion probe technique demonstrated that the asymptomatic female carrier had an 8% mutant allele fraction in DNA derived from peripheral leukocytes. This mother had 7 children, 4 of whom

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Doublecortin
Microtubule

were affected and all four affected siblings carried the mutation. Functional study showed that the mutant doublecortin protein had a significant reduction of its ability to bind microtubules.

Significance: Low level mosaicism could be a cause of inherited risk from asymptomatic parents for *DCX* related lissencephaly-subcortical band heterotopia spectrum. This is particularly important in terms of genetic counselling for recurrent risk of future pregnancies. The reduced binding affinity of mutant doublecortin may contribute to developmental malformation of the cerebral cortex.

1. Introduction

X-linked lissencephaly/subcortical band heterotopia (OMIM#300067) is a rare neuronal migration disorder where affected males have anterior predominant lissencephaly and affected females have subcortical band heterotopia (SBH, which is also called double cortex).[1–3] Clinically, affected individuals presented with refractory epilepsy and intellectual disability. Genetic variations in *DCX* genes including deletions, nonsense, frameshift and missense mutations have been reported in both sporadic and familial cases with this syndrome.[4,5]

The *DCX* gene is located on the X chromosome, encoding for a microtubule associated protein doublecortin, which is involved in neuronal migration during brain development.[6,7] The majority of DCX mutation are *de novo* and approximately 20% are inherited from the mother. Recently, low level mosaicism has been identified as an under-recognized cause of apparent "*de novo*" germline mutations.[8–10] Here, we reported a novel mutation inherited from an asymptomatic female carrier with low-level mosaicism which traditional Sanger sequencing failed to identify. With molecular inversion probe technique, we were able to quantify the mutation load and confirmed that the mutation was inherited from the mother of this family. Using cellular and biochemical assays, we also demonstrated that this novel mutation affected its binding ability to microtubules.

2. Methods

2.1. Standard protocol approvals, registration and patient consents

The study was approved by the institutional review board of Chang Gung Memorial Hospital, and written informed consents were obtained for all participants.

2.2. Participants and clinical assessment

The family was identified through the genetic epilepsy study program in Kaohsiung Chang Gung Memorial Hospital. All participants of the family were interviewed by the investigator (M.H.T) to obtain detailed clinical information. All medical records, electroencephalogram (EEG) and imaging studies were sourced and reviewed.

2.3. DNA sequencing

After informed consents were given, DNA was extracted from peripheral leukocytes using QIAGEN DNA extraction kit (QIAGEN, Valencia, CA). All coding exons and flanking intronic regions of *DCX* gene were amplified and bi-directionally sequenced on the ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA) using the Big Dye kit (PerkinElmer, Waltham, MA).[11] The potential functional impact of mutation was predicted using Polymorphism Phenotyping 2 (Polyphen-2) (http://genetics.bwh.harvard.edu/pph2/), Protein Variation Effect Analyzer (PROVEAN) (http://provean.jcvi.org/index.php) and Mutation Taster (http://www.mutationtaster.org). Segregation analysis was performed in the family members where DNA samples were available.

2.4. Allele specific PCR and molecular inversion probes technique

In order to examine the hypothesis of low-level mosaicism in the unaffected mother, we performed allele-specific polymerase chain reaction (PCR) with primers designed for the mutant allele. The allelic primers were (i) DCX4-WT forward primer 5′-AAATTTCGCTATGCTCAG**TA**-3′, (ii) DCX4-mutant forward primer 5′-AAATTTCGCTATGCTCAG**TG**-3′ and (iii) DCX4 reverse primer 5′-CCAACATTATAAGCCCTTGA-3′. The PCR mixture contained 10 μl GoTaq Green Master Mix reagent (PROMEGA), 200 nM of each primers, 1 μl patient DNA and added H_2O to 20 μl. PCR was performed with amplification protocol consisting of 1 cycle at 94 °C for 5 min, 30 cycle at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, and then 72 °C for 7 min. Then the PCR products were detected by gel electrophoresis.

To quantify the level of mutated allele, we further used molecular inversion probes (MIPs) technique to capture exon 4 of *DCX* in technical duplicates as previously described.[12]

2.5. Plasmids

For observation of doublecortin location with tubulin in cells, we used EGFP-DCX plasmid, a gift from Joseph Gleeson (Addgene plasmid ID: 32852).[13] The mutation p.Asp262Gly of

EGFP-DCX was introduced by site-directed mutagenesis kit (Agilent technologies, Santa Clara, USA). The oligonucleotide used was 5′-CCTGAAAAATTTCGCTATGCTCAGGGT-GATTTTTCTCTGGATG-3′.

For protein purification, we used EGFP-DCX as template to subclone the wild-type GST-DCX and GST-DCX p.Asp262Gly by PCR with the following primers: forward 5′-CCGGAATT-CATGGAACT TGATTTTGGA-3′; Reverse 5′-CCGCTCGAGTTA-CATGGAATCACCAAGCG-3′. The PCR fragment was cloned into PGEX4T-1 vector (GE healthcare, Piscataway, USA). All of the constructs were verified by Sanger sequencing (Genomics, Taiwan).

2.6. *Cell culture and transfection*

hTERT-immortalized retinal pigment epithelial cell line, hTERT RPE-1 were maintained in Dulbecco's modified Eagle's medium (DMEM) with F12 supplement and 10% fetal bovine serum. Transfected cells were plated on coated glass coverslides. 3×10^4 cells/well were seeded in 24-well plates and 500 ng plasmid was transfected into cells with Lipofectamine3000 reagent (Invitrogen).

2.7. *Immunostaining*

After 24 h transfection, RPE-1 cells were fixed with 4% paraformaldehyde for 10 min at room temperature and washed with 1X PBS 3 times for 5 min each time. Cells were then permeabilized with 0.2% Triton X-100 made in 1X PBS for 5 min and washed 3 times with 1X PBS for 5 min each time. After blocked with 5% BSA and 10% goat serum in 1X PBS, cells were incubated with mouse anti alpha-tubulin monoclonal antibody (1:100 66031-1-Ig, Proteintech, Chicago, USA) diluted in PBS containing 3% BSA at 4 °C overnight. Cells were washed 3 times and stained with Alexa Fluor® secondary antibodies goat anti mouse 546 (1:500, A11003, Invotrogen). The images were acquired using a confocal microscope (LSM700) equipped with a 100X oil objective lens and analyzed with Zen 2012 SP2 (Zeiss) analysis software.

2.8. *Protein purification*

Wild-type GST tagged DCX and DCX p.Asp262Gly plasmids were transformed to *E. coli* strain BL21. Colonies were inoculated into 3 ml of LB medium contain antibiotics and grown overnight at 37 °C with shaking at 225 rpm. The culture was then diluted 1:100 to 100 ml of LB medium and grown to an A600 of OD 0.3–0.6. GST fusion protein expression was induced by adding 0.2 mM of IPTG for an additional 3 h. Cells were lysed by B-PER reagent (Thermo Scientific). Lysate was incubated with Glutathione Sepharose™ 4B (GE healthcare, Piscataway, USA) at 4 °C overnight. Then bead-bound protein was eluted with reduced glutathione (Sigma). Protein quantity was checked with BSA standard by SDS-PAGE.

2.9. In vitro *protein pull down assay*

Mouse brain extract were prepared in lysis buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA; 0.5% NP-40). Purified GST-DCX proteins were quantified with BSA standard by SDS-

Table 1 – Clinical phenotype of the family with DCX p.Asp262Gly mutation.

	II-7	II-2	II-4	III-5	III-1	I-2
Gender	Male	Female	Female	Female	Female	Female
Seizure onset	Infancy	15 years old	Childhood	Childhood	10 years old	–
Seizure type	Focal clonic ± BCS Drop attacks	Focal ± BCS	Focal ± BCS	Focal ± BCS	Focal ± BCS	–
EEG	Bilateral frontal independent focal epileptiform discharges with occasionally bilateral synchrony	Bilateral theta slowing	Focal epileptiform discharges	Left posterior temporal focal spikes	Right frontal and central focal epileptiform discharges	Normal
Brain MRI	Anterior pachygyria and subcortical band heterotopia	Subcortical band heterotopia over bilateral frontal lobe	Subcortical band heterotopia over bilateral frontal lobe	Bilateral parietal and frontal subcortical band heterotopia, decreased gyration and thickening of frontal lobe	Subcortical band heterotopia over right frontal lobe	Normal
Other medical conditions	Profound ID, presenile cataract, staphyloma	Moderate ID, depression	Moderate ID	Moderate ID, mood disorder	Moderate ID	–
AEDs	CLO, LTG, VPA, TPM	VPA, LEV, OXC	LTG, VPA, ZNS	LTG, VPA, VGB	LTG, LEV	–
Mutation	Hemizygous p.Asp262Gly	Heterozygous p.Asp262Gly	Heterozygous p.Asp262Gly	Heterozygous p.Asp262Gly	Heterozygous p.Asp262Gly	Low level 8% mosaicism

BCS, bilateral convulsive seizures; CLO, clonazepam; Asp, aspartic acid; Gly, glycine; ID, intellectual disability; LEV, levetiracetam; LTG, lamotrigine; OXC, oxcarbazepam; TPM, topiramate; VGB, vigabatrin; VPA, valproate; ZNS, Zonisamine.

PAGE. Brain lysate (400ug) were incubated with 10 ug of GST-DCX at 4 °C overnight. Samples were centrifuged at 10,000 rpm for 5 min at 4 °C. Beads were washed with 1 ml RIPA buffer three times. After resuspended in 20ul cold PBS and adding 5X sample buffer, beads were boiled and ran in a SDS-PAGE gel. Immunoblot was performed with mouse monoclonal anti-alpha tubulin antibody (1:10000 66031-1-Ig, Proteintech, Chicago, USA). Image J (NIH, USA) was used to quantify the relative amount of proteins.

3. Results

3.1. *Familial and genetic study results*

The 36-year-old male proband presented with refractory seizures since infancy with various focal seizure types (Table 1). He also had profound intellect disability and could only speak single words. EEG showed independent focal epileptiform discharges over bilateral anterior quadrants. Brain MRI study showed anterior predominant fronto-parietal pachygyria (Fig. 1A). When his family history was reviewed, his three elder sisters also had refractory focal epilepsies and milder intellect disability. Brain MRI of the affected female members showed relative normal gyration pattern with a thin layer of subcortical band heterotopia (Fig. 1B). The daughter of the eldest sister was also affected with focal refractory seizures and intellectual disability. The mother of the proband did not have epilepsy and her brain MRI image appeared normal (Fig. 1C).

A novel missense mutation in exon 4 (c.785A > G, p.Asp262Gly, NM_178153) of *DCX* gene was identified by Sanger sequencing of DNA from the male proband. The mutation segregated in the affected members of the family (Fig. 1D). *In silico* prediction by several paradigms (Mutation Tasting, Polyphen2, and PROVEAN) demonstrated that the mutation was likely disease-causing (Fig. 2B). The mutation was present in all affected individuals and absent in the available non-affected siblings. Although Sanger sequencing of the unaffected mother showed no p.Asp262Gly mutation (Fig. 2A), we presumed that the mother could be a somatic mosaicism carrier. We performed allele specific PCR and demonstrated that the mutation was indeed present in the asymptomatic mother (Fig. 2C). We further quantified the mutation load in the carrier with molecular inversion probe technique followed by massive parallel sequencing. The mutant "C" allele was presented in about 8% of all reads (1291 out of total 16,148 reads; Fig. 2D).

3.2. *Functional study*

In order to evaluate the subcellular localization of the mutant doublecortin, we expressed EGFP-tagged DCX and its mutant form p.Asp262Gly in cultured cells. RPE-1 cells were

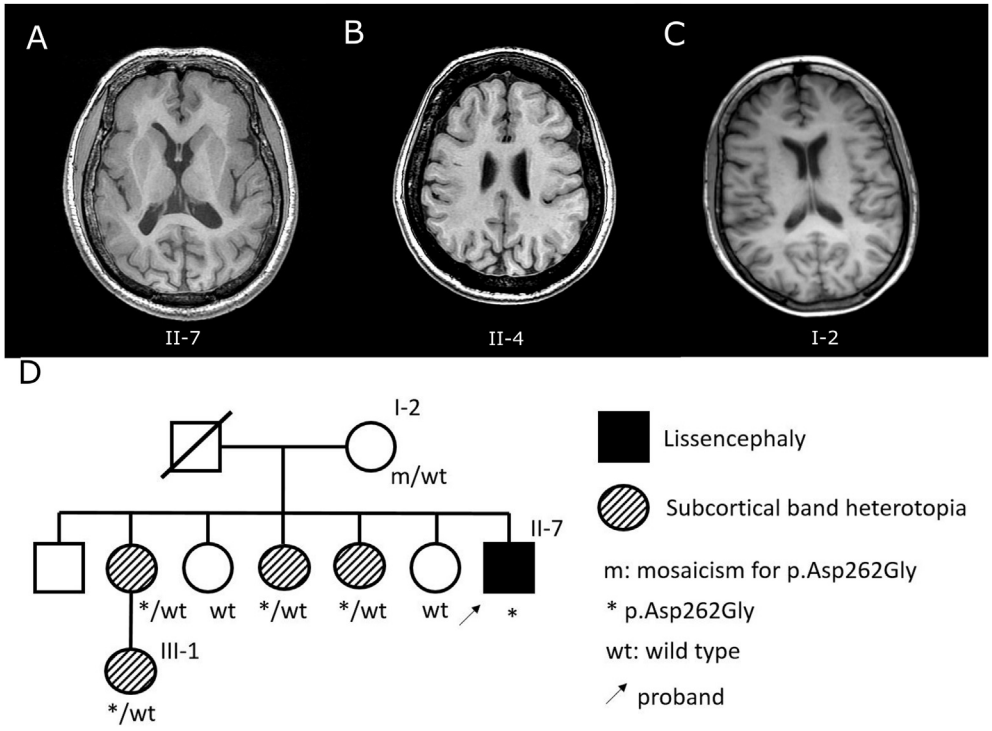

Fig. 1 – MRI and pedigree of X-linked lissencephaly/subcortical band heterotopia family. (A) Brain MR image of the affected male proband (II-7) showed thickening of grey matter and smooth reduced gyration of fronto-parietal lobes with relative preserved tempero-occipital lobes, which is consistent with anterior predominant pachygyria. There were also mild subcortical band heterotopia over frontal polar and parietal area. (B) Brain MR image of affected female siblings (II-4) showed subcortical band heterotopia over bilateral frontal and parietal lobes. (C) MR image of unaffected mother showed normal morphology (I-2). (D) The pedigree of X-linked lissencephaly/subcortical band heterotopia family.

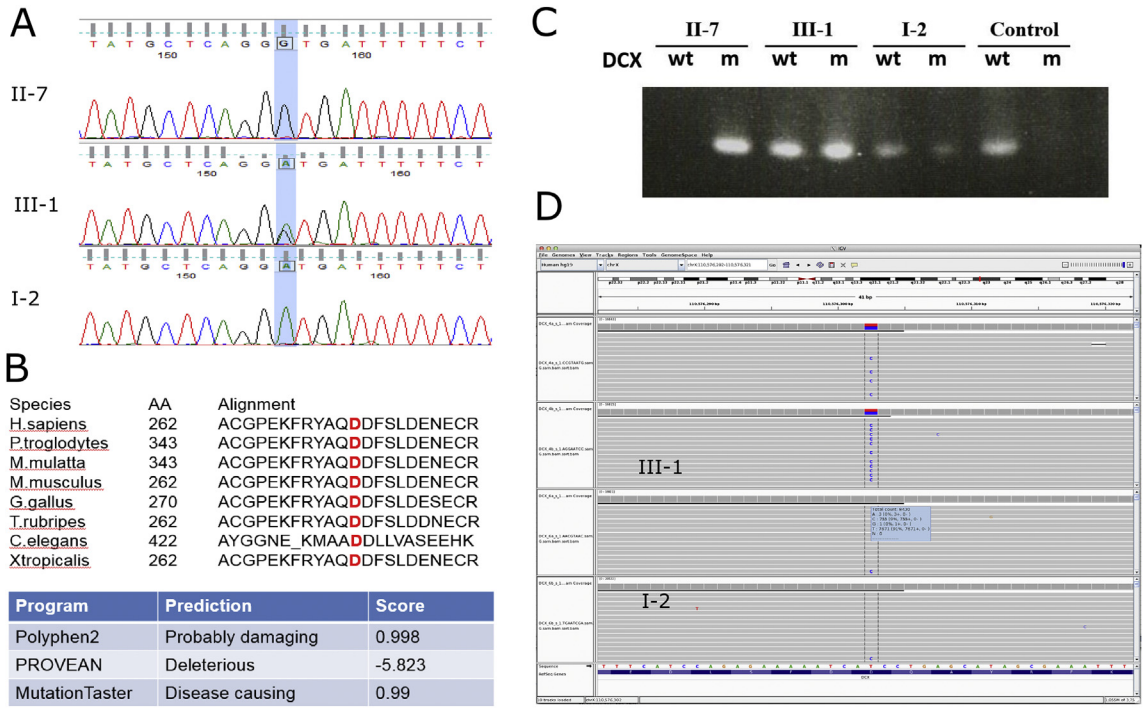

Fig. 2 – Molecular genetic analysis of the *DCX* p.Asp262Gly mutation. (A) The mutation (c.785A > G, p.Asp262Gly) of *DCX* gene showed hemizygous mutation of the affected male proband (II-7), heterozygous mutation of the affected female niece (III-1). Sanger sequencing showed no mutation in the unaffected mother (I-2). (B) The mutation site is located at an amino acid residue highly conserved from P. Troglodytes to X. Tropicalis. In silico prediction shows that the mutation is potentially pathogenic using Polyphen2, PROVEAN and Mutation Taster software. (C) Allele specific PCR demonstrated that the unaffected mother (I-2) had the mutant allele. (D) Mapped reads revealed low level mosaicism (8%) in the unaffected mother detected by molecular inversion probes techniques (shown in technical duplicates using Integrated Genomic Viewer), which was not detectable by Sanger sequencing.

transfected with plasmids expressing EGFP-DCX or EGFP-DCX p.Asp262Gly and counter stained with tubulin antibody. We found that in cells with moderate expression of EGFP-DCX or EGFP-DCX p.Asp262Gly, they both co-localised with microtubules (Supp Fig. 1A). Consistent with previous findings, overexpression of EGFP-DCX led to the induction of microtubule bundles in the periphery of the cell soma.[14] Overexpression of the EGFP-DCX p.Asp262Gly also resulted in very similar phenotype (Supp Fig. 1).

In order to evaluate the properties of the mutant doublecortin, protein pull down assay was performed *in vitro*. Mouse brain lysate was incubated with purified GST-tagged wild type doublecortin or p.Asp262Gly mutant bound to glutathione beads. Microtubules bound to doublecortin were co-sedimented by centrifugation and analysed with Western blotting. We found that there was a 70% decrease in the amount of tubulin protein bound to mutant doublecortin compared to the wild type (Fig. 3). These findings suggested that the mutant form has much lower binding ability to microtubules.

4. Discussion

We report a family with X-linked lissencephaly and SBH caused by a novel DCX mutation c.785A > G (p.Asp262Gly) inherited from a low-level (~8%) mosaic asymptomatic mother using molecular inversion probes (MIPs) technique. We further confirmed that the novel mutation affected the ability of doublecortin binding to microtubule.

DCX is located on the X chromosome and is typically inherited from affected mothers who are heterozygous for the mutation, although paternal transmission from an affected father who is a somatic mosaic carrier has been reported recently.[15] The mosaic *DCX* mutation or deletion in male patients usually caused SBH rather than lissencephaly.[9,15–19] In a large French study of lissencephaly-SBH spectrum, 88.5% of female patients and 23% of male patients were found to have *de novo* mutations of *DCX*.[4] Recently, parental low-level mosaicism has been identified as an under-recognized risk of genomic disorders.[5] Using molecular inversion probes and next generation sequencing techniques, we confirmed that the novel *DCX* mutation was inherited from the asymptomatic mother who has ~8% of the mutated allele not detected by Sanger sequencing. It has been suggested that the severity of clinical phenotypes is associated with the percentage of *DCX* mosaicism in the tissue.[9,17,18] Consistent with this hypothesis, our female carrier had no seizures with normal intellect and normal brain MRI. Our study suggests that some of the previously reported "*de novo*" mutations in apparent sporadic cases might be inherited from low-level mosaicism parents. A similar phenomenon has recently also been observed in

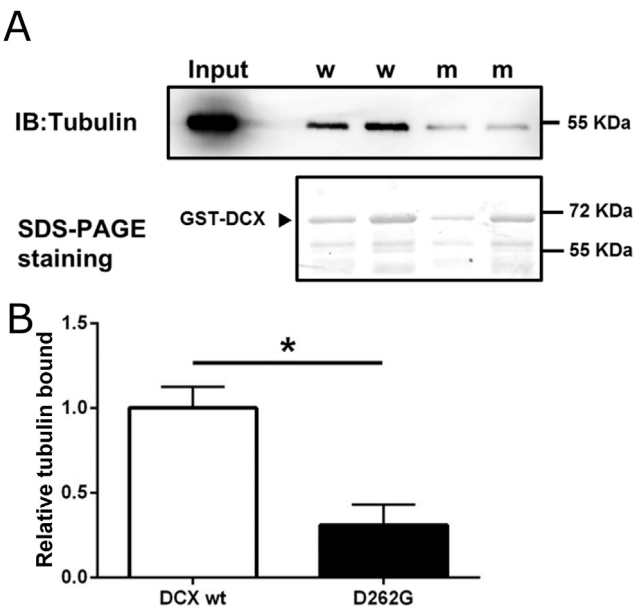

Fig. 3 – *In vitro* microtubule binding ability of wild type doublecortin and the p.Asp262Gly mutant. (A) Mouse brain lysate was incubated with wild type (w) or p.Asp262Gly mutant (m) doublecortin bound to beads at 4 °C overnight. The upper panel shows the amount of tubulin protein pulled down by centrifugation. The lower panel shows the amount of protein bound to the beads as normalization reference. The amount of tubulin bound to DCX mutant was clearly decreased. Two independent experiments are shown. IB: Immunoblot. (B) Quantification of tubulin bound to wild type or mutant doublecortin. There was a 70% decrease in the amount of tubulin bound to DCX p.Asp262Gly mutant compared to the control (0.31 ± 0.12 *vs.* 1.01 ± 0.13, n = 3, $p = 0.017$, student's *t* test).

patients of Dravet syndrome with ~10% of "*de novo*" mutations arising from asymptomatic parents.[20] This is particularly of clinical significance, especially in terms of genetic counselling about the recurrent risk of future pregnancies.

Doublecortin binds to microtubules, which play a critical role in the neuronal migration during embryonic and postnatal development.[4,21] The novel p.Asp262Gly mutation is located in the C-DC domain of the dourblecortin protein, which is one of the two evolutionarily conserved functional domains. Previous studies have implied that mutations in the C-DC domain tended to have a less severe phenotype compared to those in the N-DC domain.[22] Importantly, the 262 aspartic acid residue is highly conserved among different species, indicating its importance to the protein function. Our biochemical assay revealed that the mutant doublecortin exhibited a significant decrease in the binding ability to microtubules. Interestingly, a nearby missense *de novo* mutation p.Asp263Gly has been reported in a male patient with lissencephaly. 3D structural modelling predicts that p.Asp263Gly mutation is located on the surface of C-DC domain and may destabilize the protein.[4] It is possible that p.Asp262Gly mutation in our family may affect tubulin binding through direct interaction or destabilization of DCX protein. The decrease in binding affinity may in turn contribute to pathogenicity in the patients. Previous studies have shown that doublecortin function through binding to microtubules, enhancing microtubule polymerization, facilitating microtubule bundling and regulating growth cone function.[14,23–27] The deletion analysis showed that the first 213 amino acids of DCX are sufficient to bind microtubules.[23] It is possible that C-DC domain may have regulatory roles in microtubule binding or other microtubule mediated function. Missense mutations are clustered within both N-DC and C-DC domains in similar frequency in patients, which underscores the importance of both domains in microtubule related function.[15] Further studies of the effects of C-DC domain mutations on microtubule polymerization or bundling might unravel its additional roles than the well-studied N-DC domain.

In conclusion, we identified a novel DCX mutation inherited from maternal low-level mosaicism, which was not captured by Sanger sequencing. The mutation caused reduced binding ability to microtubules, which may contribute to the brain structural alteration in the patients.

Potential conflicts of interest

All authors report no potential conflicts of interest relevant to this work.

Authors' contribution

Dr PWK, HYC contributed to the design, acquisition, analysis and interpretation of the functional data for the manuscript.

Dr CTM, HCM performed the MIP experiments and subsequent variant calling to quantify mosaicism.

Dr YCC, WJL, SWL, TYF contributed to the acquisition and interpretation of the data and revising the manuscript for intellect content.

Dr MHT, JWT contributed to the design and conceptualization of the study; analysis and interpretation of the data; drafting, revising and final approval of the manuscript for intellectual content.

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejpn.2016.05.010.

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