**TUBA1A mutation can cause a hydranencephaly-like severe form of cortical dysgenesis**

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TUBA1A mutations cause a wide spectrum of lissencephaly and brain malformations. Here, we report two patients with severe cortical dysgeneses, one with an extremely thin cerebral parenchyma apparently looking like hydranencephaly and the other with lissencephaly accompanied by marked hydrocephalus, both harbouring novel de novo missense mutations of TUBA1A. To elucidate how the various TUBA1A mutations affect the severity of the phenotype, we examined the capacity of the mutant protein to incorporate into the endogenous microtubule network in transfected COS7 cells by measuring line density using line extraction in an immunofluorescence study. The mutants responsible for severe phenotypes were found to incorporate extensively into the network. To determine how each mutant alters the microtubule stability, we examined cold-induced microtubule depolymerisation in fibroblasts. The depolymerisation of patients’ fibroblasts occurred earlier than that of control fibroblasts, suggesting that microtubules bearing mutated tubulins are unstable. Both mutations are predicted to participate in lateral interactions of microtubules. Our data suggest that the TUBA1A mutations disrupting lateral interactions have pronounced dominant-negative effects on microtubule dynamics that are associated with the severe end of the lissencephaly spectrum.

In the past two decades, it has become evident that the genes encoding cytoskeletal proteins are important in the developing brain1. Their importance was initially inferred from the identification of genes encoding microtubule-associated proteins (MAPs), such as LIS1 (also known as PAFAH1B1) and DCX, which are mutated in the lissencephaly spectrum2. Several years later, new disorders associated with mutations in genes encoding for α- or β-tubulin were described3–10. The α- and β-tubulins are the major components of microtubules and are characterised by variable isotypes whose expressions are spatially

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and temporally regulated. Mutations in a number of neuronally expressed tubulin genes are associated with a spectrum of cortical development malformations commonly referred to as tubulopathies. These disorders are caused by mutations in TUBA1A, TUBB2B, TUBB3, TUBB, TUBB4A, TUBB2A, and TUBG1 genes. These mutations thought to involve varying degrees of abnormal neuronal proliferation, migration, and postmigrational development that result in a large spectrum of malformations, including lissencephaly, pachygyria, polymicrogyria, and microcephaly.

Microtubules are ubiquitous structural components that contribute to the cytoskeleton, cilia, flagella, axon fibres, and mitotic spindles. Microtubules are dynamic polymers consisting of tandem repeats of α- and β-tubulin heterodimers, which assemble in a head-to-tail fashion at the growing ends of microtubules. Each microtubule is constructed from 13 laterally connected protofilaments of repeating tubulin heterodimers; lateral interactions between each microtubule form the hollow and cylindrical microtubule body.

TUBA1A gene encoding α1a-tubulin is expressed in almost all post-mitotic neurons throughout neuronal development. TUBA1A-related cortical dysgenesis typically shows a posteriorly predominant lissencephaly with cerebellar hypoplasia (LCH), dysmorphic basal ganglia, thin or absent corpus callosum, congenital microcephaly, ventricular dilatation, and abnormalities of the hippocampus and brainstem. However, the clinical phenotypes caused by TUBA1A mutations vary considerably. Recently, TUBA1A mutations have also been described in perisylvian asymmetrical polymicrogyria, polymicrogyria-like cortical dysplasia, and microsclerencephaly in foetal cases. The clinical manifestations of affected patients often include congenital microcephaly, severe intellectual disability, neurodevelopmental delay with diplegia or tetraplegia, and epilepsy.

In our study, we performed whole-exome sequencing of two patients with severe cortical dysgenesis. One patient had an extremely thin cerebral parenchyma apparently looking like hydranencephaly, whereas the other had lissencephaly accompanied by marked hydrocephalus. We identified two novel de novo heterozygous TUBA1A mutations, c.190 C>T (p.R64W) and c.74 G>T (p.C25F). In addition, we performed a functional assay of the mutant proteins to determine why these patients show more severe phenotypes than patients with classical lissencephaly.

Results
Patients' characteristics. Patient 1 (NCU_F41) was a 3-year-old girl. She was born at a gestational age of 37 weeks by caesarean section. Her parents were healthy and unrelated. Her elder sister was also healthy and had normal development. Her mother was referred to our hospital for foetal growth restriction, microcephaly, and marked ventricular dilatation of her foetus on ultrasonography from 28 weeks of gestation. At patient delivery, the amniotic fluid was excessive but the placenta and umbilical cord were normal. Her Apgar scores were 3 and 5 at 1 and 5 min, respectively. She could not breathe spontaneously and needed mechanical ventilation. Her birth weight was 2116 g (−2.0SD), head circumference was 33 cm (−0.2SD), and body length was 44 cm (−1.8SD). She had microcephaly, microphthalmos, widely spaced eyes, and micrognathia. Truncal hypotonia with spastic tetraplegia was evident and her digital joints were contractured.

An ophthalmologic examination revealed bilateral optic nerve hypoplasia. Foetal MRI at 28 weeks of gestation and brain MRI at 6 days after birth revealed an extremely thin cerebral parenchyma, hypoplastic brain stem, and agenesis of the cerebellum and corpus callosum (Fig. 1a–d). Test results for toxoplasma, rubella, cytomegalovirus, and herpes simplex (TORCH) infections were negative. Her karyotype was normal 46, XX. After birth, she presented with focal clonic seizures, sometimes with oxygen desaturation. Her electroencephalogram showed extremely poor background activities and focal rhythmic delta waves during the seizures. As the seizures were treated with phenobarbital, they were partially controlled.

At the age of 1 month, a tracheotomy and tracheal separation were performed because of her recurrent aspiration pneumonia. A gastrostomy and fundoplication were also done at the same time. Because of the agenesis of the pituitary, trichlormethiazide for central diabetes insipidus and hydrocortisone for hypothyroidism were needed. Since her head circumference had been gradually enlarged, a ventriculoperitoneal shunt was placed at the age of 7 months to control the head growth and to assist in nursing care. At 3 years of age, she had spastic tetraplegia and no definite awareness of her environment.

We performed whole-exome sequencing of peripheral blood DNA from the patient and both her parents (Supplementary Fig. S1a). A de novo heterozygous c.190 C>T (p.R64W) variant was identified in TUBA1A. Subsequent Sanger sequencing confirmed the presence of this variant in the patient 1 and absence in the genomes of both her parents (Supplementary Fig. S2). The c.190 C>T variant was predicted to be damaging by both PolyPhen-2 and SIFT. No potentially pathogenic variants related to malformations of cortical development were identified in any other genes in patient 1 (Supplementary Table S1).

Patient 2 (K3373) was a 2-year-old boy. Ventricular dilatation was identified in the foetal period. His parents were healthy and unrelated. His elder sister was also healthy and had normal development. He was born at a gestational age of 39 weeks by vaginal delivery. His Apgar scores were 8 and 9 at 1 and 5 min, respectively. His birth weight was 2792 g (−0.9SD), head circumference was 33 cm (−0.2SD), and body length was 49.5 cm (+0.3SD). After birth, he was diagnosed with lissencephaly (Fig. 1e–h). He could breathe and swallow by himself. At the age of 8 months, he began suffering from epileptic spasms,
which could be controlled with sodium valproate and zonisamide. At 2 years of age, he had spastic tetraplegia and was unable to roll over. Although he would not make eye contact and did not speak, he could react to sound and had a smile when his mother called his name.

We performed whole-exome sequencing of peripheral blood DNA from the patient and both his parents (Supplementary Fig. S1b). A \textit{de novo} heterozygous c.74 G>T (p.C25F) variant was identified in \textit{TUBA1A} and confirmed by Sanger methods (Supplementary Fig. S2). This variant was not detected in the genomes of both his parents by Sanger sequencing. The c.74 G>T variant was predicted to be damaging by both PolyPhen-2 and SIFT. There were no potentially pathogenic variants related to malformations of cortical development in any other genes in patient 2 (Supplementary Table S1). Both \textit{TUBA1A} mutations of patients 1 and 2 are located at the amino acids which are conserved across many species (Supplementary Fig. S1c).

\textbf{Structural modelling of \textit{TUBA1A} mutations.} Both \textit{TUBA1A} mutations reported here are located in the N-terminal domain (Supplementary Fig. S1d) and are predicted to be associated with lateral interactions between microtubules (Fig. 2a–c). According to structural studies conducted using cryoelectron microscopy, protofilaments in microtubules are primarily connected between the M loops and the H1’-S2 and H2-S3 loops\textsuperscript{15}. R64 is located on the H1’-S2 loop of \(\alpha\)-tubulin (Fig. 2c), which participates in lateral interactions. R64 forms hydrogen bonds with the surrounding residues, E3, F53, and S54 (Fig. 2d). These hydrogen bonds may be involved in the structural stability and/or flexibility of the lateral interactions. Therefore, the R64W mutation is predicted to directly disrupt the lateral interaction. C25 is located on the boundary between helix H1 and the H1-H1’ loop of \(\alpha\)-tubulin (Fig. 2c), and this residue faces the luminal side of microtubules (Fig. 2a). The H1-H1’ loop appears to support the H1’-S2 loop and the H2-S3 loop to enhance lateral interactions\textsuperscript{15}. Therefore, the C25F mutation may secondarily compromise lateral interactions.

\textbf{\textit{TUBA1A} mutants alter the ability of \(\alpha\)-tubulin to incorporate into the microtubule network.} We examined the ability of the \textit{TUBA1A} mutants to incorporate into the endogenous microtubule network (Fig. 3). We generated constructs designed to express \textit{TUBA1A} mutants upon transfection of COS7 cells. The constructs were for wild-type, p.R64W, and p.C25F, as well as p.R402C, which is a recurrent \textit{TUBA1A} mutation that expresses the phenotype of classical lissencephaly, similar to \textit{LIS1} mutations\textsuperscript{23}. Transfected cells were examined by immunofluorescence using an anti-FLAG antibody to detect the expression of the transgene and an anti-\(\alpha\)-tubulin antibody to detect the overall microtubule network.
At 24 h post-transfection, we found that FLAG-tagged wild-type TUBA1A was visualised as lines (Fig. 3a,B) and colocalised with the α-tubulin cytoskeleton (Fig. 3a,C), suggesting that FLAG-tagged wild-type TUBA1A could incorporate into the microtubule network. On the other hand, we found that FLAG-tagged mutant TUBA1A was visible not only as lines, but also as puncta that were diffusely distributed throughout the cytoplasm (Fig. 3a,E,H,K). The linear staining merged with α-tubulin, but the puncta did not colocalise with α-tubulin (Fig. 3a,F,I,L), suggesting that some of the mutant TUBA1A protein could not incorporate into the microtubule network. We observed more incorporated FLAG-tagged TUBA1A protein with R64W and C25F transfection than with R402C. To exclude the influence of the acidic charges of the FLAG tag, we also examined the incorporation of the Myc-tagged TUBA1A protein. Consistent with the data of the FLAG-tagged protein, the incorporation into the microtubule network of Myc-tagged mutant TUBA1A was less than wild-type (Supplementary Fig. S4).

To quantify the incorporation of mutant protein in each transfected cell, we determined the linear staining of FLAG-tagged TUBA1A of each cell by using the ImageJ KBI Line Extract plug-in for line extraction24 (Supplementary Fig. S3a). These lines indicated the microtubule network of incorporated FLAG-tagged TUBA1A. After line extraction, we measured the total length of the lines and calculated the line density of each cell. The microtubule density of FLAG-tagged TUBA1A for each mutant transfection was analysed.

We found that the microtubule density of FLAG-tagged mutant TUBA1A was significantly lower than that of FLAG-tagged wild-type TUBA1A (Fig. 3b,A). Among TUBA1A mutants, the microtubule density of R64W was the highest, whereas that of R402C was the lowest. We also calculated the microtubule density of α-tubulin in the same manner (Fig. 3b,B). We found that the microtubule density level followed the same order as that of FLAG-tagged TUBA1A. Therefore, the amount of the overall microtubule network tended to depend on the amount of incorporated overexpressed TUBA1A protein. These data indicated that R64W tended to permit higher levels of microtubule incorporation than C25F and R402C. Because there were no significant differences in the mean relative FLAG intensities, the expression levels

Figure 2. Three-dimensional mapping of TUBA1A-mutated residues in a microtubule structure. (a) End-on view of a microtubule structural model. A microtubule consists of 13 longitudinal protofilaments that are connected via lateral interactions. α-tubulin molecules are blue, β-tubulin molecules are white, R64 residues are red, and C25 residues are orange. (b) Side view of a microtubule. C25 residues are not shown in this view because they are located on the luminal side of a microtubule. (c) Higher resolution image of the lateral interaction between the α-tubulin molecules. Both light blue and dark blue are α-tubulin molecules. The M loop is gold, the H1′-S2 loop is red, the H1-H1′ loop is green, and the helix H1 is yellow. The R64 and C25 residues have side chains in this figure. (d) The R64 residue forms hydrogen bonds with surrounding residues, E3, F53, and S54.
of FLAG-tagged protein in the analysed cells were thought to be similar among wild-type and mutants (Supplementary Fig. S3b).

To assess microtubule dynamics in transfected COS7 cells, we examined repolymerisation after cold induced depolymerisation (Supplementary Fig. S5). There were significantly less cells containing the asters of α-tubulin in the cases of R64W transfection than wild-type (Supplementary Fig. S5c).

**TUBA1A mutants alter microtubule stability.** We investigated microtubule behaviour in patients’ fibroblasts to assess the effects of these mutations on the microtubule stability (Fig. 4). Microtubules of fibroblasts start to depolymerise when they are incubated on ice. We examined the cytoskeleton morphology and the depolymerised tubulin in the fibroblasts by immunofluorescence staining of α-tubulin (Fig. 4a). We first showed by RT-PCR that TUBA1A was actually expressed in fibroblasts of the patients and control fibroblasts (control_1) (Supplementary Fig. S6). We defined a cell that contained no linear staining of α-tubulin at 400× magnification to be a completely depolymerised cell. We compared the percentage of completely depolymerised cells between patients’ fibroblasts and control ones when they were incubated on ice for 0, 5, 10, 15, and 20 min (Fig. 4b). There were no completely depolymerised control or mutant cells after 0 and 5 min of cold treatment (Fig. 4a,A–F). After 10 min of cold treatment, we found that the percentage of completely depolymerised cells from the R64W and C25F patients was more than four times that of the control (Fig. 4a,G–I,b). The differences for comparison R64W and C25F cells with control cells were statistically significant (p = 5.6 × 10⁻²⁴ and p = 5.8 × 10⁻¹⁴, respectively, Fig. 4b). This suggested that the depolymerisation of patients’ fibroblasts occurred sooner than the control ones. There were almost no differences in the percentage of depolymerised cells after 20 min of cold treatment (Fig. 4a,M–O,b). Repolymerisation of the microtubules could occur in control and mutant...
fibroblasts after treatment on ice for 20 min and subsequently at 37 °C for 15 min (Fig. 4a,P–R). These data suggested that mutated microtubules are less stable than normal ones.

To assess an underlying mechanism by which TUBA1A mutations lead to cortical dysgeneses, we examined the mitosis and the migration of the patients’ fibroblasts (Supplementary Fig. S7, S8). There were no significant differences between the patients’ and control fibroblasts both in the mitosis and in the migration.

Discussion

In our present study, we identified two novel heterozygous missense TUBA1A mutations in patients with severe cortical dysgeneses, one with an extremely thin cerebral parenchyma apparently looking like hydranencephaly and the other with lissencephaly accompanied by marked hydrocephalus. TUBA1A-related cortical dysgenesis typically shows a posteriorly predominant lissencephaly with cerebellar hypoplasia (LCH), dysmorphic basal ganglia, thin or absent corpus callosum, congenital microcephaly, ventricular dilatation, and abnormalities of the hippocampus and brainstem\textsuperscript{16}. Without these characteristics, it is difficult to determine whether a case with certain brain malformations is a TUBA1A-related disorder using

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**Figure 4. Microtubule behaviour and stability in patients’ fibroblasts after various periods of cold treatment.** (a) Before cold treatment, the cytoskeleton morphologies were similar among control and mutant fibroblasts (A–C). Note that fibroblasts of R64W and C25F were depolymerised after 15 min of cold treatment, whereas polymerised microtubules were still present in control fibroblasts (J–L). Scale bar, 20 μm. (b) Percentage of completely depolymerised cells after cold treatment of 0, 5, 10, 15, and 20 min. We counted completely depolymerised cells among 275–400 cells in each condition. After 10 min of cold treatment, the percentage of R64W and C25F fibroblasts showing depolymerisation was more than four times that of control fibroblasts. Asterisks indicate statistically significant differences compared with control (Fisher’s exact test and Bonferroni correction using numbers of cells with and without complete depolymerisation, *p < 0.05, **p < 0.0001).
only MRI findings. The brain MRI of our patient harbouring the TUBA1A p.R64W mutation manifested an extremely thin cerebral parenchyma with severe hydrocephalus, agenesis of the cerebellum and the corpus callosum, and hypoplastic brain stem, the most severe form of brain malformations. It looked like hydranencephaly but was distinguished from hydranencephaly by the existence of an extremely thin cerebral parenchyma. The cerebral cortex was too thin to assess the layer structure using brain MRI. Thus, our data indicate that the spectrum of TUBA1A-related brain malformations is broader than expected.

All previously reported, TUBA1A mutations have been heterozygous missense mutations23,25. The presence of missense mutations and the absence of nonsense mutations, frameshifts, or whole gene deletions suggest that the mutation results in gain-of-function or has a dominant-negative effect, rather than haploinsufficiency. Among tubulopathies, it has been proposed that the severity of nervous system impairments may depend on the relative abundance of mutant α- and β-tubulin heterodimers compared with wild-type, combined with their ability to incorporate into the microtubule cytoskeleton, which affect dynamics, motor protein, or MAP interaction in different dominant-negative fashions13.

In the case of TUBB3 mutations, R262H substitution permits much more heterodimer formation and microtubule incorporation than R262C, both in vitro and in mammalian cells. R262C results in isolated eye movement restrictions, whereas R262H causes not only severe eye movement restrictions, but also other neurological impairments and brain malformations6. In the same way, the recurrent R402H mutation in TUBA1A, which causes a more severe lissencephaly with complete agryria than R402C, produces a mutant protein that permits higher levels of heterodimer formation and microtubule incorporation than R402C23,27. We showed that R46W had the highest amounts of mutant protein incorporation into the network compared with the other mutants and caused the most severe phenotype. In the repolymerisation experiments, the expression of TUBA1A R46W protein impaired the ability of the endogenous α-tubulin to repolymerise. Thus, a greater extent of mutant protein incorporation into the endogenous microtubule network may result in more severe phenotypes in a dominant-negative fashion.

In the current study, the data of the incorporation of FLAG-tagged R402C TUBA1A was different from the previous reports23,27. The reason of the difference could be based on the position of the FLAG tag. To exclude the influence of the acidic charges of the FLAG tag, we also examined the incorporation of the Myc-tagged TUBA1A protein. Consistent with both the data of the FLAG-tagged protein, limited incorporation of the R402C mutation was observed and the data did not depend on the type of tags. According to the previous report27, the R402C mutation generated tubulin heterodimers in significantly reduced yield in vitro folding reaction. It may be consistent that the R402C mutation disrupts αβ-heterodimerisation, leading to little incorporation into the microtubule network.

Tubulin contains three separate structural domains, N-terminal, intermediate, and C-terminal28. These three domains participate in five distinct functions: heterodimer stability, longitudinal and lateral protofilament interactions, nucleotide exchange and hydrolysis, and microtubule–protein interactions13. In neurons, lateral interactions are particularly important because microtubules are arranged in dense networks and must be resistant to forces that cause bending or buckling to maintain their structural integrity13,15,29. Mutations found at positions essential for lateral interactions are predicted to impede the polymerisation and dynamic properties of microtubules, resulting in microtubules that may be relatively nondynamic or unstable and more likely to depolymerise13. In cold-induced depolymerisation of fibroblasts, it is consistent that the two TUBA1A mutants, R64W and C25F, could make microtubules less stable than those of the controls by disrupting lateral interactions.

We investigated the severity of TUBA1A-related cortical dysgeneses that are associated with lateral interactions in previous reports as well as in this report. TUBA1A mutations that primarily participate in lateral interactions include L286F20 (M loop), E55K31, T56M25, R64W (H1′-S2 loop), and L92V23 (H2-S3 loop). The patients harbouring L286F, T56M, and L92V were foetuses. Using the classification of Kumar et al.23, four mutations (L286F, T56M, R64W, and L92V) are classified in the severe lissencephaly with cerebellar hypoplasia (LCH severe group 4). On the other hand, E55K belongs to the moderate lissencephaly (LIS moderate group 1) with extreme microcephaly. There are also neighbouring loops that may support lateral interactions. The H1-H1′ loop supports the H1′-S2 loop and the H2-S3 loop, and the end of H6 and the S9-S10 loop appear to stabilise the M loops35. TUBA1A mutations that secondarily participate in lateral interactions include C25F, E27Q26 (H1′-H1 loop), Y210C27, D218Y23 (the end of H6), G366R32, A369T25, and V371E25 (S9–S10 loop). Five mutations (E27Q, Y210C, D218Y, G366R, and V371E) were classified into the LCH severe group 4 and C25F resembled the LCH severe group 4 phenotype except for milder cerebellar vermis hypoplasia. Only A369T showed central pachygyria. Therefore, many of the reported TUBA1A mutations at positions associated with lateral interactions caused severe phenotypes of brain malformations (LCH severe group 4).

In the case of β-tubulin, R62 of TUBB3 is also positioned in the H1′-S2 loop of β-tubulin and is predicted to participate in lateral interactions6. However, patients harbouring R62Q have a milder phenotype among TUBB3 mutations. As it was reported that residues involved in lateral interactions cluster in regions of divergence between species and marked difference between α- and β-tubulins28, there may be different consequences for α- and β-tubulins when lateral interactions are disrupted. We propose that the differences in clinical manifestations among tubulopathies caused by a mutated tubulin gene should be evaluated.

In conclusion, our data suggest that mutations in TUBA1A at positions essential for lateral interactions may lead to severe phenotypes of brain malformations. However, we did not show the consequences
of these mutants for neuronal developmental processes such as proliferation, migration, differentiation, and axonal guidance. In patients’ fibroblasts, the mitosis and the migration were not impaired. Further studies of each mutant affects microtubule function and how it impairs neuronal developmental processes will reveal the precise function of microtubules in normal neuronal development.

Methods

Patients. Genotyping testing was approved by the ethical committees of Fujita Health University and collaborated institutes in accordance with the principles of the Declaration of Helsinki, and the Ethical Guidelines for Human Genome/Gene Analysis Research by the Ministry of Education, Culture, Science, and Technology, the Ministry of Health, Labor, and Welfare, and the Ministry of Economy, Trade, and Industry of Japan. Blood samples from affected individuals and their parents, and skin biopsy samples were obtained with informed consent according to local institutional review board guidelines.

Whole-exome sequencing and validation. Genomic DNA was extracted from peripheral blood using the QIAamp DNA Blood Midi Kit according to the manufacturer’s instructions (Qiagen, Tokyo, Japan). Three micrograms of DNA were sheared into 150–200-bp fragments using theM220 Focused-ultrasonicator (Covaris, Woburn, MA USA). To capture the exonic DNA, we used the SureSelect XT Human All Exon V5 capture library (Agilent Technologies, Santa Clara, CA USA). We then constructed a sequence library using the SureSelect XT Target Enrichment System for the Illumina Paired-End Sequencing Library kit (Agilent Technologies) and performed DNA sequencing of 100-bp paired-end reads using the Illumina HiSeq 2000 sequencer. The sequencing data were mapped to the reference genome (GRCh37/hg19) using BWA (ver.0.6.1). Variant calling was performed using SAMtools (ver.0.1.16) and GATK (ver.1.6) software as previously reported33. To identify disease causative mutations, we excluded known variants found in public databases (dbSNP138, 1000 Genomes Project, NHBLI ESP6500, and Exome Aggregation Consortium [ExAC]) and a control in-house database, except for those also identified as pathogenic mutations in the NCBI ClinVar and HGMD databases. We focused on non-synonymous single nucleotide variants (SNVs), insertions and deletions (indels), and splice site variants. Predictions of possible impact of amino acid substitution on the structure and function by variant were performed using PolyPhen-234 and SIFT35 software. The mutations were confirmed by Sanger sequencing.

Structural modelling of TUBA1A mutations. Tubulin dimer structure (PDB ID:1JFF) docked into the density map (MT-13-3, EMDB ID: EMD-5193) using Chimera (https://www.cgl.ucsf.edu/chimera/) after the homology modelling of missing residues 35–60 in α-tubulin was kindly provided by Dr. Haixin Sui, New York State Department of Health15. Polymserised microtubule images were then generated with Chimera and MolFeat (FiatLux, Tokyo, Japan).

Transfection experiments. The full-length cDNA encoding the human TUBA1A sequence was generated by PCR using a template from placenta cDNA (Forward primer: 5′-TAAGCGGCCGCGCATG CTGAGTGCATCTCCATCCAC-3′, Reverse primer: 5′-TAACTGCAGGTATTCCTCTTCTTTCC TCACCCCTC-3′). The PCR product was cloned into the Not I and Pst I sites of the pCMV-4A vector (Agilent Technologies), which is a mammalian expression vector for tagging proteins with a C-terminal FLAG (DYKDDDDK) epitope under the control of the CMV promoter. Three mutations—p.R64W, p.C25F, and p.R402C—were generated by PCR using a template of the wild-type tubulin and SuperScript III First-Strand Synthesis System (Life Technologies). For construction of the Myc tag vectors, the Xho I and Apa I fragment of these FLAG tag constructs were replaced with a synthesized DNA encoding a Myc tag (The fragment sequence is 5′-TCAGGGACAAAAACTCATCTCGAAGAGGATCTGTAGGGCC-3′). All constructs were checked by DNA sequencing.

Constructs were transfected into COS7 cells grown on glass coverslips in Opti-MEM I using Lipofectamine 2000 (Life Technologies). At 24h after transfection, cells were fixed with ice-cold methanol and stained with a mouse monoclonal anti-α-tubulin antibody (Santa Cruz Biotechnology) and a goat polyclonal anti-DYKDDDDK antibody (Novus Biologicals) at dilutions of 1:250 and 1:5000, respectively. Secondary antibodies were donkey anti-mouse IgG Alexa Fluor 488 and donkey anti-goat IgG Alexa Fluor 594 (Life Technologies) at dilutions of 1:1000. In depolymerisation experiments of COS7 cells35, cells were incubated on ice for 30min and then restored to 37°C for 1.5 min. Cells were immediately fixed with ice-cold methanol and stained as above. For the Myc tag experiment, a rabbit polyclonal anti-Myc antibody (MBL) at dilution of 1:100 and donkey anti-rabbit IgG Alexa Fluor 594 (Life Technologies) at dilutions of 1:1000 were used.

RT-PCR. RT-PCR was done by manufacturer’s protocol. NucleoSpin RNA II (MACHEREY-NAGEL) and SuperScript III First-Strand Synthesis System (Life Technologies) were used. The primer sequences of TUBA1A and HPRT are shown in the Supplementary Figure S6.

Depolymerisation experiments of fibroblasts. Fibroblasts were derived from skin biopsies taken from the patients with p.R64W and p.C25F mutations and control people. The depolymerisation experiments were done as previously described37. Briefly, fibroblasts (passage 3) grown on glass coverslips in
IMDM containing 10% foetal bovine serum, penicillin 50 U/ml and streptomycin 50 μg/ml were incubated for 0, 5, 10, 15, and 20 min on ice and fixed. Repolymerisation experiments of fibroblasts were performed by incubating cells on ice for 20 min and then restoring them to 37 °C for 15 min. Cells were stained with a mouse monoclonal anti–α-tubulin antibody used at a dilution of 1:100. The secondary antibody was donkey anti-mouse IgG Alexa Fluor 488 used at a dilution of 1:1000.

**Mitotic index and mitotic spindle formation.** Fibroblasts (passage 5) grown on glass coverslips were fixed with ice-cold methanol and stained with a mouse monoclonal anti–α-tubulin antibody and a rabbit polyclonal anti-phospho Histone H3 (pH3) (Ser10) antibody (MILLIPORE) at dilutions of 1:250 and 1:100, respectively. Secondary antibodies were donkey anti-mouse IgG Alexa Fluor 488 and donkey anti-rabbit IgG Alexa Fluor 594 (Life Technologies) at dilutions of 1:1000. The pH3 signal-positive mitotic fibroblasts were counted from at least 830 total cells.

**In vitro scratch assay.** The in vitro scratch assay was done as previously described. For each image, distances between one side of scratch and the other were measured using ImageJ and 50 readings of distances were measured for each sample.

**Image acquisition.** Slides were observed under a fluorescence microscope (Axio Imager M1; Carl Zeiss) equipped with a digital camera (AxioCam HRc; Carl Zeiss). COS7 cells and fibroblasts were observed at 630× and 400× magnifications, respectively. In vitro scratch assay, dishes were observed under Axiovert 200M (Carl Zeiss) equipped with a digital camera (AxioCam MRm; Carl Zeiss) and fibroblasts were observed at a 100× magnification. Images were captured with the same settings using AxioVision 4.8 software (Carl Zeiss).

**Quantification of microtubule density and relative FLAG intensity.** Quantification of microtubule density was performed as previously described. For each COS7 cell, a region of interest (ROI) was manually assigned along the edge of the cell by the free hand tool in ImageJ. We then calculated the area and the mean intensity of the ROI by using the ImageJ ROI manager. We used the KBI Line Extract plug-in for line extraction. The parameters were giwsIter 5, mdnmsLen 20, pickup above 0.0, shaven Len 5, and del Len 5. The total length of the extracted lines was calculated by the KBI Line Feature plug-in for the α-tubulin staining and the FLAG staining. The total length of the extracted lines in a selected cell divided by the area of the cell gave the microtubule density. These plug-ins are available as part of the KBI ImageJ plug-in package (http://basezawa.ib.k.u-tokyo.ac.jp/zp/Kbi/ImageJ/KbiPlugins). The relative FLAG intensity was determined in ImageJ by subtracting the mean red signal intensity of an untransfected cell from that of a transfected cell. The untransfected cell was in the same image as that of the transfected cell.

**Statistical analysis.** One-way ANOVA and Tukey’s post-hoc analysis and a chi-square test were performed using IBM SPSS Statistics Version 22. Fisher’s exact test and the Bonferroni correction were performed using R software. We considered p < 0.05 to be significant after correction.

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**Author Contributions**

S.Y., M.T. and N.F. performed all experiments except whole-exome sequencing. F.M. performed whole-exome sequencing and the analysis. M.K., N.O., T.T., M.Y., Y.K., K.K. and S.S. participated in whole-exome sequencing. N.I., H.Y., S.K. and J.N. contributed materials. I.Y. constructed the microtubule structural model (Fig. 2). S.Y., N.I. and H.K. wrote the manuscript. All authors reviewed the manuscript.

**Additional Information**

Supplementary information accompanies this paper at http://www.nature.com/srep

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