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Investigation of the most common clinical and imaging findings and the role of tubulin genes in the etiology of malformations of cortical development*

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Background and aim: The number of reports on the role of tubulin gene mutations (TUBA1A, TUBB2B, and TUBB3) in etiology of malformations of cortical development has peaked in recent years. We aimed to determine tubulin gene defects on a patient population with simple and complex malformations of cortical development, and investigate the relationship between tubulin gene mutations and disease phenotype.

Materials and methods: We evaluated 47 patients with simple or complex malformations of cortical development, as determined by radiological examination, for demographic features, clinical findings and mutations on TUBA1A, TUBB2B, and TUBB3 genes.

Results: According to the magnetic resonance imaging findings, 19 patients (40.5%) had simple malformations of cortical development and 28 (59.5%) patients had complex malformations of cortical development. Focal cortical dysplasia was the most common simple malformation, lissencephaly was the most common coexisting cortical malformation, and corpus callosum anomalies were the most common coexisting extracortical neurodevelopmental abnormalities. None of the patients had genetic alterations on TUBA1A, TUBB2B, and TUBB3 genes causing protein dysfunction. On the other hand, the frequencies of some polymorphisms were higher when compared to the literature.

Conclusion: It is crucial to identify the etiology in patients with malformations of cortical development in order to provide appropriate genetic counseling and prenatal diagnosis. We consider that multicenter studies with higher patient numbers and also including other malformations of cortical development-related genes are required to determine underlying etiological factors of malformations of cortical development patients.

Key words: Cortical dysplasia, tubulinopathies, TUBA1A, TUBB2B, TUBB3

1. Introduction
Malformations of cortical development (MCD) are an important cause of refractory epilepsy, intellectual disability, developmental delay, and neurological deficits. Studies in recent years have shown that mutations on genes encoding i) different tubulin isotypes (TUBA1A, TUBA8, TUBB2A, TUBB2B, TUBB3, TUBB, TUBG1), ii) microtubule-associated proteins (LIS1, DCX, KIF2A, KIF5C), or iii) microtubule-based motor proteins (kinesin, dynein) play a role in etiology of MCD [1].

Tubulinopathies include a broad spectrum of brain malformations (microlissencephaly, classical lissencephaly, polymicrogyria, schizencephaly, corpus callosum agenesis or hypoplasia, dysmorphic basal ganglia, brain stem and cerebellar hypoplasia, ventriculomegaly, and vermian hypoplasia) and clinical findings (cognitive and/or motor impairments, epilepsy, congenital microcephaly). Furthermore, congenital fibrosis of the extraocular muscles and progressive sensorimotor polyneuropathy could be seen in specific TUBB3 and TUBB2 mutations. Tubulinopathies generally are inherited in an autosomal dominant manner. On the other hand, some reported tubulinopathy families with TUBA8 mutations had autosomal recessive inheritance1 [2,6].
In this study, we aimed to determine TUBA1A, TUBB2B, and TUBB3 gene defects in simple MCD and complex MCD (with additional cortical and/or extracortical neurodevelopmental malformations) patient group with unknown etiology, and explore relationship between disease phenotype and clinical progression in patients with tubulin gene mutations.

2. Materials and methods

2.1. Patients

Patients referred to our department from three pediatric hospitals for radiologically diagnosed MCDs with unidentified etiology for a 6-month period were included in this study. All participants were below 18 years of age. Detailed information was given to all participants and their relatives. Written informed consents were obtained from the all participants and/or their parents. The study protocol received approval by Dokuz Eylül University Non-Invasive Researches Ethical Review Board and the approval code is 2015/12-51.

2.2. Data collection

Information obtained from examinations and medical records were recorded in data collection forms. All patients were examined by a pediatric neurologist and clinical geneticist. The following information were recorded; personal information, prenatal history, history of epilepsy (age of onset, type of seizures, EEG findings, response to antiepileptics), motor development stages, head circumference, examination notes, imaging results, hearing test results, genetic test results, and pedigree. Cognitive function and motor development were evaluated using the Wechsler Intelligence Scale of Children-Revised (WISC-R) and the Denver Developmental Screening Test, respectively. All MR images were evaluated by two pediatric neuroradiologists. The classification of the MCD subtype was made according to the Barkovich classification system. MCD associated with additional cortical or extracortical malformations were classified as complex MCD.

2.3. Mutation screening

For DNA isolation, 5 mL of peripheral blood sample was collected. DNA was extracted from lymphocytes using standard methods (High Pure PCR Template Preparation Kit, Roche™). All coding exons and exon-intron junctions of TUBA1A, TUBB2B, and TUBB3 genes were amplified with polymerase chain reaction (PCR) using Helix Amp™ Hot-Taq Polymerase [Ver 2.0] (with dNTP Mix) (NanoHelix) for TUBA1A gene part 4b and TUBB3 gene part 4a, and Helix Amp™ Ready-2X Multiplex version 2.0 PCR mix (NanoHelix) for the rest of the other parts. Protocols were performed according to the standards provided by the kit. Primers and amplicon lengths were listed in Table 1. Amplifications were performed using a Mastercycler Gradient-Eppendorf® thermal cycler. Thermocycling conditions were presented in Table 2. PCR products were verified by 2% agarose gel electrophoresis and ethidium bromide staining.

BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used for the second PCR step. Second PCR conditions were as follows: Initial denaturation step at 96 °C for 1 min, followed by 25 cycles including denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s, and extension at 60 °C for 4 min. Then, Zymo Research DNA Sequencing Clean-up Kit™ was used to purify the PCR products. Protocols were performed according to the standards provided with the kits. Purified PCR products were analyzed using ABI 3130 capillary electrophoresis system (Applied Biosystems, Thermo Fisher Scientific).

CLC Genomics Workbench 3.6.5 sequencing software (Qiagen) was used for analysis. ENST00000301071.11, ENST00000259818.7 and ENST00000554444.5 transcripts in the ENSEMBLE database were used as reference sequences. All variations were interpreted using mutation and single nucleotide polymorphism (SNP) databases and in silico programs (Human Genome Mutation Database, ClinVar, National Center for Biotechnology Information/SNP, Ensembl, PolyPhen 2, and Mutation Taster). Each variation was confirmed by bidirectional sequencing. All variants were classified according to 2015 ACMG standards and guidelines for the interpretation of sequence variants, and they were described according to the nomenclature recommended by the Human Genomic Variation Society. In case of novel genetic variations, the patients’ parents were also taken into consideration.

3. Results

A total of 47 patients (25 females, 22 males) with simple or complex MCD, as determined by radiological studies and with no diagnosis of recognizable genetic syndromes, were analyzed for TUBA1A, TUBB2B, and TUBB3 mutations. The mean age of patients was 6.3 years (range 1 month and 17 years). Prenatal history was remarkable in 7 (14.9%)
patients. Consanguineous marriage was reported in 8 (17%) families.

In the present study, the most common clinical findings were intellectual disability (83%, n = 39) and epilepsy (55.3%, n = 26) followed by microcephaly (29.8%, n = 14), macrocephaly (14.9%, n = 7), hypotonia (14.9%, n = 7), strabismus (14.9%, n = 7), and abnormal cerebellar findings (10.6%, n = 5). EEG abnormalities were reported in 24 (51.1%) patients. The most seen seizure type was generalized tonic clonic seizures (48.1%, n = 13). Clinical and cranial MRI findings were presented in Table 3.

Simple MCD was reported in 19 (40.5%) patients. Focal cortical dysplasia (n = 6, 12.8%) was the most common simple MCD subtype. Five patients (10.6%) had heterotopia, three patients (6.4%) had schizencephaly, three patients (6.4%) had polymicrogyria, and two patients (4.2%) had lissencephaly. Twenty-eight patients (59.5%) were classified as complex MCD. Lissencephaly was the most common coexisting cortical malformation, whereas corpus callosum anomalies were the most common coexisting extracortical neurodevelopmental abnormalities. The classification of MR findings was shown in Table 4.

Sequencing analysis demonstrated no pathogenic alterations on exons and/or exon-intron junctions. Variations were detected in these three genes summarized

| Gene | Exon/Part | Forward primer (5’->3’) | Reverse primer (5’->3’) | Amplicon length (bp) |
|------|-----------|------------------------|------------------------|---------------------|
| TUBA1A | 1 | TTCTAACCCCAGTCCCCCTT | CCTCGCCAGAGAGCTTAC | 507 |
| | 2 | GTGTAGTGCTGGGATAG | AGAACATGAGGGAGGAGGA | 509 |
| | 3 | GTGCTGGGACAGGAGGC | AAATAACAGTTCAATTCTGTTGTA | 361 |
| | 4a | TTTTGGGTTTTAAAATTC | GAGCCTCAATATCGAGGTTTCT | 487 |
| | 4b | CCCTGGAGACACTCTTGA | AAATGGCAGCTTGGCTG | 949 |
| | 4c | GACCAAAGGGTACCATCCAG | AAATGGCAGCTTGGCTG | 522 |
| TUBB2B | 1 | CACCCCTCTTGCATAAAAGG | GCCAAAGTCACCTCCTAGCC | 375 |
| | 2 | ATTCATGTTGAGGCTTGGC | GCAGGGAAAGGGAGAAG | 238 |
| | 3 | CTGGTTTGGGGAACAC | CTGGCAATCAGACCTTCTCA | 288 |
| | 4a | TGGGCTGGGTTGGAGGGTTAAGGT | ACCCTCTTCTGAGGACATGCG | 768 |
| | 4b | ACCCGAGAGATGTTCGACTC | GCCAGGGTATGCTGCCG | 59 |
| TUBB3 | 3 | GCCTCCTTAGGATGAGAGGAGG | GTCTGGCCATCAGAGCTTGA | 259 |
| | 4a | AGAGACAGAAAGGGGAGG | GGGTCACTCAGAGAAGTA | 877 |
| | 4b | GTTCATGAGGACAAGATGA | GGGTCTAGACATGCTTGGCT | 540 |

Table 2. Thermocycling conditions of TUBA1A, TUBB2B, and TUBB3 genes.

| Stage | Temperature | Time | Number of cycles |
|-------|-------------|------|------------------|
| Initial denaturation | 94 °C | 15 min | 1 |
| Denaturation | 94 °C | 30 s | 35 |
| Annealing | 60 °C | 45 s | 35 |
| Extension | 72 °C | 45 s | 35 |
| Final extension | 72 °C | 7 min | 35 |
| For TUBA1A part 4b and TUBB3 part 4a | Initial denaturation | 95 °C | 15 min | 1 |
| Denaturation | 95 °C | 20 sec | 35 |
| Annealing | 60 °C | 40 sec | 35 |
| Extension | 72 °C | 1 min | 35 |
| Final extension | 72 °C | 7 min | 35 |
Table 3. Clinical and cranial MRI findings

| Patient No | Sex | Age      | PH | HC | ID/MDD | Epilepsy/ST | EEG Abn. | NE | OE | CM | MR imaging     |
|------------|-----|----------|----|----|--------|-------------|----------|----|----|----|----------------|
| 1          | M   | 8 years  | -  | N  | +      | -           | -        | N | -  | -  | FCD, PFA       |
| 2          | F   | 12 years | -  | Mac.| +      | +/GTC       | +        | N | -  | -  | FCD, Het.     |
| 3          | M   | 9 months | -  | N  | +      | -           | -        | DTR↑, hypo. | N | -  | Lis., CCA     |
| 4          | F   | 6 years  | -  | N  | +      | -           | -        | N | -  | -  | FCD           |
| 5          | F   | 9 years  | -  | N  | N      | -           | N        | N | -  | -  | FCD           |
| 6          | F   | 7 months | +  | N  | N      | -           | -        | N | -  | -  | FCD           |
| 7          | M   | 4 years  | -  | N  | +      | +/Focal     | +        | N | Strab. | -  | FCD, PFA     |
| 8          | F   | 2 years  | -  | -  | -      | -           | -        | N | -  | -  | Het.          |
| 9          | M   | 8 years  | -  | N  | +      | +/SGTC      | +        | N | -  | -  | FCD, CCA      |
| 10         | F   | 10 years | -  | Mic.| +      | +/GTC       | +        | Spas. | N | -  | FCD, CCA     |
| 11         | F   | 11 years | -  | N  | N      | -           | N        | N | +   | Lis., CCA     |
| 12         | F   | 12 years | -  | N  | N      | +/-Myoclonic| -        | N | Ptosis | +  | Het.          |
| 13         | M   | 3 months | -  | N  | NA     | -           | -        | Hypo. | N | -  | Lis., PMG     |
| 14         | F   | 7 years  | -  | Mic.| +      | +/GTC       | +        | DTR↑,Pr, Spas. | N | -  | FCD           |
| 15         | F   | 5 years  | +  | Mac.| +      | +/GTC       | +        | DTR↑   | N | -  | FCD, PFA     |
| 16         | M   | 15 years | -  | Mic.| +      | +/GTC       | +        | N | +   | FCD           |
| 17         | M   | 8 years  | -  | Mac.| +      | +/-Absans   | +        | N | -  | -  | FCD, Het.     |
| 18         | F   | 8 years  | -  | N  | +      | +/-GTC      | -        | DTR↑, Spas. | N | -  | Sch.          |
| 19         | M   | 13 years | -  | N  | N      | +/-GTC      | +        | Hypo. | N | -  | Het., PFA    |
| 20         | M   | 3 months | -  | N  | +      | +/-SGTC     | +        | PR    | Nys. | -  | PMG           |
| 21         | M   | 13 years | -  | Mic.| +      | -           | +        | Spas. | N | -  | Lis., PFA    |
| 22         | M   | 17 years | -  | N  | +      | +/-GTC      | +        | N | Strab. | -  | Lis., Sch.   |
| 23         | M   | 12 years | -  | Mic.| +      | +/-Clonic   | +        | Hypo. | N | -  | FCD           |
| 24         | F   | 1 months | -  | Mic.| NA     | +/-SGTC     | +        | DTR↑,PR, Spas. | N | -  | Lis., PMG     |
| 25         | F   | 11 years | +  | N  | N      | +/-GTC      | +        | DTR↑, Spas. | N | -  | FCD, CCA     |
| 26         | M   | 6 years  | -  | N  | +      | -           | DTR↑     | N | -  | -  | Sch.          |
| 27         | M   | 12 years | -  | N  | +      | +/-Focal    | +        | N | -  | -  | Het.          |
| 28         | F   | 10 years | -  | Mac.| +      | -           | -        | N | -  | -  | Het.          |
| 29         | F   | 4 years  | +  | Mic.| +      | -           | -        | N | -  | -  | Lis.          |
| 30         | F   | 3 years  | -  | N  | +      | -           | -        | Hypo. | N | +  | Sch.          |
| 31         | F   | 4 years  | +  | Mic.| +      | +/-Focal    | +        | DTR↑,PR, Spas. | N | -  | PMG           |
| 32         | F   | 7 years  | -  | Mic.| +      | +/GTC       | +        | Spas. | N | -  | Het., CCA    |
| 33         | M   | 1 months | +  | Mac.| NA     | -           | -        | N | -  | -  | Het., CCA, PFA |
| 34         | F   | 13 years | -  | N  | +      | -           | -        | N | N   | +  | FCD, PFA     |
| 35         | M   | 3 years  | +  | Mac.| +      | +/-SGTC     | +        | N | -  | -  | Het.          |
| 36         | M   | 3 years  | -  | Mic.| +      | -           | -        | N | N   | +  | Sch., CCA    |
| 37         | F   | 9 years  | -  | N  | +      | +/-Focal    | +        | DTR↑,PR, Spas. | Strab. | -  | Het., CCA, PFA |
| 38         | F   | 2 years  | -  | N  | +      | -           | -        | N | Strab. | -  | Het., PFA    |
| 39         | M   | 6 years  | -  | N  | +      | -           | -        | N | -  | -  | Het., CCA    |
| 40         | F   | 1 years  | -  | Mic.| +      | +/GTC       | Hypo.    | Strab. | -  | Het., CCA, Lis. |
| 41         | F   | 1 years  | -  | N  | +      | -           | DTR↑,PR, Spas. | Strab. | -  | FCD           |
in Table 5. On TUBA1A gene, linkage disequilibrium for rs1056875 A>G and rs697624 G>C polymorphisms was found in 28 patients. For TUBA1A gene rs1056875 A>G SNP, 19 patients (40.4%) had AA genotype, 23 patients (49%) had AG genotype, and 5 patients (10.6%) had GG genotype. In our patient group, the frequency of A allele was 65% (n = 61), and the frequency of G allele was 35% (n = 33). For TUBA1A gene rs697624 G>C SNP, 19 patients (40.4%) had GG genotype, 23 patients (49%) had GC genotype, and 5 patients (10.6%) had CC genotype. In our patient group, the frequency of G allele was 65% (n = 61), and the frequency of C allele was 35% (n = 33).

On TUBA1A gene, rs199717430 C>T (11:c.226+10 C>T) was detected on a single allele in a 12-year-old male patient with linear heterotopia. The patient’s parents were also analyzed due to lack of sufficient population studies. The results showed that the patient’s healthy father with normal brain MRI also carried the same variation in heterozygous state.

4. Discussion
Tubulin mutations are seen in 1–13% of all MCDs [7]. In this study, we investigated TUBA1A, TUBB2B, TUBB3 mutations on 47 simple and complex MCD patients. We did not find any pathogenic variations, whereas some polymorphisms were more frequently detected than reported before.

Mutations on tubulin genes cause tubulinopathies, which include varying clinical findings (cognitive and motor impairments, refractory epilepsy, congenital microcephaly) and a broad spectrum of brain malformations (microlissencephaly, classical lissencephaly, polymicrogyria, schizencephaly, corpus callosum agenesia or hypoplasia, dysmorphic basal ganglia, brain stem and cerebellar hypoplasia, ventriculomegaly, and vermis hypoplasia) [7,9].

In our study, the most common clinical findings were intellectual disability (83%, n = 39) and epilepsy (55.3%, n = 26) followed by microcephaly (29.8%, n = 14), macrocephaly (14.9%, n = 7), hypotonia (14.9%, n = 7), strabismus (14.9%, n = 7) and abnormal cerebellar findings (10.6%, n = 5). EEG abnormalities were reported in 24 (51.1%) patients. The most seen seizure type was generalized tonic clonic seizures (48.1%, n = 13).

The most common type of MCD in present study was focal cortical dysplasia followed by heterotopia, lissencephaly, schizencephaly, and polymicrogyria. Twenty-eight patients (59.5%) were classified as complex MCD. Lissencephaly was the most common coexisting...
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Cortical malformation, while corpus callosum anomalies were the most common coexisting extracortical neurodevelopmental abnormalities.

DNA sequencing was used to screen TUBA1A, TUBB2B, and TUBB3 mutations. None of the patients had any genomic alterations on exon sequences and exon-intron junctions, which could disrupt protein function (mutation). We found linkage disequilibrium for rs1056875 A>G and rs697624 G>C polymorphisms on TUBA1A. Similarly, Rosendahl et al. [10] explored TUBA1A mutations in 46 patients with lissencephaly, and reported rs1056875 A>G and rs697624 G>C single nucleotide polymorphisms (SNPs) showing linkage disequilibrium.

rs199717430 C>T SNP (11:c.226+10 C>T), which is located on intron 2 splice+10 of TUBA1A and creates a missense intronic variant was detected on a single allele in a 12-year-old male patient with linear heterotopia. Population studies have shown that the frequency of T allele is less than 0.1% in all populations. This variation is recorded as a “likely benign allele” in databases (ClinVar). Due to the lack of sufficient population studies, the patient’s parents were also analyzed. The patient’s father also had the same variation in a heterozygous state, and he had normal phenotype. Considering that i) the patient’s father has a normal phenotype, ii) the variation is located on the intron, and iii) autosomal dominant inheritance of TUBA1A mutations, this variation is not likely to be responsible for the observed phenotype.

In conclusion, the patient group in the present study represents a significant patient population. We did not find any pathogenic variation in any of three genes, and it is likely that small sample size and the high number of genes responsible for MCDs are main reasons for this observation. Furthermore, the rate of consanguineous marriages in our study (17%) was higher than the rates of developed countries (<5%) [11]. Consanguineous marriages are associated with an increased risk for autosomal recessive diseases; therefore, the high risk of having an autosomal recessive disease is another reason to consider. We think that multicenter studies with higher patient numbers and also gene panels including other MCD-related genes are required to determine underlying etiological factors of MCD patients.

Acknowledgment
We thank the patients participating in the present study.

Conflict of interest
The authors declare no conflict of interest.

Table 5. Variations were detected in three genes.

| Gene     | TUBA1A          | TUBB2B          | TUBB3           |
|----------|-----------------|-----------------|-----------------|
| Variation|                 |                 |                 |
| rs1056875 A>G | rs697624 G>C | rs199547345 C>T |                 |
| (11:c.288 A>G) | (11:c.453 G>C) | (7:c.718 C>T)  |                 |
| (p.K96K)    | (p.S151S)      | (p.L240L)      |                 |
| Genotype   | AA AG GG GG GC CC CT TT | CC CT TT | CC CT TT |
| %          | 40.4 49 10.6 40.4 49 10.6 98 2 0 | 80.9 19.1 0 98 2 0 | 93.6 6.4 0 93.6 6.4 0 98 2 0 |
| n          | 19 23 5 19 23 5 46 1 0 | 38 9 0 46 1 0 | 44 3 0 44 3 0 46 1 0 |
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