INTRODUCTION

Tuberous sclerosis complex (TSC), first described in 1862,\(^1\) is a clinically and phenotypically variable disease affecting many different tissue and organ systems. Patients may manifest skin lesions, or have anomalies of the brain, kidney, heart, and lung. The incidence of TSC is about 1 in 6000 live births.\(^2\) Defects in the \(TSC1\) (*605284) or \(TSC2\) (*191092) genes encoding the components of the Tuberous Sclerosis Complex are responsible for the disease. Therefore, consideration of \(TSC1/TSC2\) pathogenic variations is recommended in the updated diagnostic criteria of Tuberous Sclerosis Complex.

Aims: To present the \(TSC1/TSC2\) screening results of a mixed patient population as well as possible new variants in 23 cases from 20 different families who were referred to our Genetic Diseases Diagnosis Center with the signs and symptoms of Tuberous Sclerosis Complex.

Study design: Retrospective, cross-sectional study.

Methods: Germline \(TSC1/TSC2\) variants were screened in DNA samples extracted from peripheral blood samples of 23 patients from 20 unrelated families using targeted high-throughput sequencing and multiplex ligation-dependent probe amplification methods. The variants identified were classified according to ACMG 2015 guidelines.

Results: In total, 5 different pathogenic/likely pathogenic changes have been defined. All these pathogenic/likely pathogenic variants were located in the \(TSC2\) gene. Three of the pathogenic/likely pathogenic variants were novel. Two patients who are twin sisters were found to have \(TSC2/PKD1\) contiguous deletion syndrome. One of the 3 novel variants was a mosaic in-frame deletion. We did not identify any pathogenic variants of the \(TSC1\) gene.

Conclusion: The novelty of most of the variants found, including a mosaic likely pathogenic variant, and the presence of a large genomic rearrangement, supports the importance of a comprehensive approach in analyzing \(TSC1/TSC2\) genes. Genetic diagnosis should be performed with caution, considering the possibility of mosaic variants with low allelic fractions.

The TSC, formed by the hamartin protein encoded by the \(TSC1\) gene and the tuberin protein encoded by the \(TSC2\) gene, has essential roles in metabolism, protein synthesis, cell growth, and angiogenesis. Loss of function of \(TSC1/TSC2\) tumor suppressor genes results in uncontrolled cell growth and tumors due to overactivation of mTORC1 (mechanistic target of rapamycin complex 1).\(^3\) According to updated diagnostic criteria, TSC can be diagnosed by clinical features, as well as by the presence of a heterozygous
pathogenic variant in the TSC1 or TSC2 genes. The clinical diagnostic criteria include 2 different subgroups called major and minor features. The major features include: angiofibroma, fibrous cephalic plaque, hypomelanotic macules, ungual fibromas, cortical dysplasia, shagreen patch, multiple retinal hamartomas, subependymal nodules or giant cell astrocytoma, cardiac rhabdomyoma, lymphangioleiomyomatosis (LAM), and angiomylipomas. The minor features include confetti-like skin lesions, dental pits, intraoral fibromas, retinal achromic patch, multiple renal cysts, and nonrenal hamartomas. Two major features or 1 major feature with 2 or more minor features are needed for a definite clinical diagnosis. One major feature together with 2 or more minor features is required for “possible diagnosis.” Beyond these minor and major clinical criteria, there is a wide range of signs and symptoms that have been related to abnormalities of TSC listed in the Human Phenotype Ontology (HPO) and Online Mendelian Inheritance in Man databases. For a definite diagnosis of TSC, identifying a TSC1 or TSC2 pathogenic variation is sufficient. Therefore, genetic analysis of TSC1 and TSC2 genes has great importance in diagnosing TSC.

Genetic analysis of TSC1/TSC2 genes has been challenging for a long time due to the large size of the TSC2 gene. High-throughput sequencing (HTS) analysis, which has become increasingly common in both research and clinical practice in the last decade, provides essential advantages in diagnosing TSC due to its ability in simultaneous analysis of large genes. Determining the genetic background of the disease is critical for precise genetic counseling. In about 3% of the patients, the underlying molecular defect may be gross deletions/duplications. These rearrangements are more frequent in the TSC2 than in the TSC1 gene and may involve both the TSC2 and the PKD1 genes, adjacent genes located on chromosome 16. The multiplex ligation-dependent probe amplification (MLPA) method is a well-known method to detect large genomic deletions/duplications in a targeted manner. Therefore, it is a valuable method for detecting TSC1/TSC2 deletions/duplications.

This study aimed to present the results of a comprehensive genetic analysis of 23 patients from 20 different families referred to our Genetic Diseases Diagnosis Center with a clinical suspicion of TSC. By reporting 3 novel and 1 known causative variant defined by HTS and a TSC2/PKD1 contiguous deletion syndrome characterized by MLPA, this study emphasizes the importance of using both HTS and MLPA in the genetic analysis of TSC1/TSC2 genes.

### MATERIAL AND METHODS

The high-throughput sequencing and MLPA analysis results of the TSC1 and TSC2 genes of 23 patients (8 males, 15 females, mean age: 10.6 ± 11.6, Table 2) from 20 different families who were tested in the Genetic Diseases Diagnosis Center between February 2013 and January 2019 were included in this study. Written informed consent was obtained from the patients or the legal guardians of the patients younger than 18 years old. The Institutional Ethical Board approved this study with the decision number BAEK-2021/45.

### Whole Blood Collection and DNA Isolation

Peripheral blood samples were collected from patients with informed consent forms for genetic investigation. DNA was isolated from EDTA blood samples by the BioRobot EZ1 system (Qiagen Hilden, Germany), according to the manufacturer’s instructions. The Qubit 2.0 fluorometer (Invitrogen, Life Technologies) was used for quality and quantity control of isolated DNA samples prior to library construction.

### TABLE 2. Clinical/Phenotypic and Demographic Features of our Study Population

| Clinical/phenotypic and demographic features | N (%) |
|--------------------------------------------|-------|
| Renal abnormalities                        | 8 (34.78) |
| Cranial abnormalities                      | 11 (47.82) |
| Dermatologic findings                      | 10 (43.47) |
| Rhabdomyoma                                | 3 (13.04) |
| Epilepsy                                   | 12 (51.17) |
| Gender                                     |       |
| Female                                     | 15 (65.21) |
| Male                                       | 8 (34.78) |
High-Throughput Sequencing Library Construction

The primers targeting the coding regions of \textit{TSC}1 and \textit{TSC}2 genes with 20 bp intron padding were designed with the Ion AmpliSeq Designer (Life Technologies). Amplicon libraries were constructed with a mix including 4 \(\mu\)L of 5X Ion AmpliSeq HiFi mix, 10 \(\mu\)L of multiplexed primer pool, 10 ng of gDNA per reaction, and 4 \(\mu\)L of molecular-grade water. The PCR mixes were subjected to thermal cycling conditions as follows: at 99°C for 2 minutes; 99°C for 15 seconds, 60°C for 4 minutes (19 cycles); and a final hold at 10°C. Adapters and barcodes (Ion Xpress, Life Technologies) were ligated to the amplicons including partially-digested primers, and were prepared as described in the Ion AmpliSeq library preparation protocol. The Ion One Touch 2 System and the Ion One Touch ES Instruments (Life Technologies) were used for the production of Enriched, template-positive Ion Sphere Particles. Sequencing of enriched particles was performed on the PGM (Life Technologies) with 314 and 318 chips, according to the user guide for the Ion PGM sequencing 200 kit v2.2 (Life Technologies).

Torrent Suite Software v.5 (Life Technologies) was used for raw data processing, alignment, and variant calling. The hg19 human reference genome (Genome Reference Consortium GRCh37) was used as the reference genome.

Coverage analysis control was performed with the Ion Reporter’s coverage analysis plugin. At least 20x coverage for targeted bases was accepted as reliable for variant calling. Samples under this threshold were repeated. Ion Reporter v5.0 software was used for annotating and filtering variants.

The amplicons of \textit{TSC}1 and \textit{TSC}2 genes of each sample were also checked manually with the help of the IGV 2.4.8 program.\textsuperscript{17} For segregation analyses, primers covering the variant region of interest were designed in our laboratory. Amplicons obtained after the polymerase chain reaction were subjected to dideoxy chain-termination reaction using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s protocol and sequenced with the ABI3130 XL (Applied Biosystems, Foster City, CA, USA) automatic capillary system.

Multiplex Ligation-Dependent Probe Amplification

The P124-C3 and P337-B1 MLPA probe mixes (MRC-Holland, Amsterdam, the Netherlands) were used to test possible deletions/duplications of the \textit{TSC}1 and \textit{TSC}2 genes. Ligation and amplification of ligated probes were performed following the manufacturer’s protocol, and the products were separated on the ABI 3130 XL (Applied Biosystems, USA) capillary electrophoresis system. The sizes of the fragments were determined using the GeneMapper (Applied Biosystems, USA) program, and the results were analyzed with the Coffalyser software (MRC Holland).

In Silico Predictions and Variant Classification

The variants identified were classified according to ACMG 2015 guidelines\textsuperscript{18} considering population frequencies, in gnomAD,\textsuperscript{19} ClinVar,\textsuperscript{20} and dbSNP\textsuperscript{21} databases, HGMD\textsuperscript{22} database accessions, Leiden Open Variation accessions,\textsuperscript{23} previous reports, and in silico prediction tools. CADD\textsuperscript{24} and MutationTaster\textsuperscript{25} were used to predict the pathogenic potential of all rare/novel variants. PhyloP\textsuperscript{26} was considered for conservation scores. MaxEntScan\textsuperscript{27} and GeneSplicer (http://www.cceb.umd.edu/software/GeneSplicer/gene_spl.shtml) predictions were applied to the splicing variants.

Statistical Analysis

Descriptive analyses of variants and patient demographics were used for the frequency distributions. Mean age and standard deviations were calculated with R open software.

RESULTS

In total, 4 different pathogenic/likely pathogenic variants have been determined by HTS (Table 3). Two patients who were twin sisters had \textit{TSC}2/\textit{PKD}1 contiguous deletion syndrome with a heterozygous loss encompassing \textit{TSC}2 33-42nd exons and \textit{PKD}1-30/\textit{PKD}1-40 gene regions, defined by MLPA.

Three out of 4 variations defined by HTS were novel variants that were not previously reported elsewhere (Table 4). The \textit{TSC}2 (NM_000548): c.1000_1002delGTG (p.Val334del) in-frame deletion defined in patient 4 was a mosaic variant with a ratio of 27%. Sequencing analysis revealed decreased peak heights, confirming the mosaicism of this variant which was initially determined via HTS. This mosaic patient’s parents did not have the \textit{TSC}2 c.1000_1002delGTG (p.Val334del) deletion, as expected. The parents of patient 4 were also available for segregation analysis, which confirmed that the \textit{TSC}2 c.4253dupC (p.Gln1419ThrfsTer105) variant, previously reported in the LOVD database, was a de novo variant. We did not define any pathogenic/likely pathogenic variants of the \textit{TSC}1 gene in our patients.

DISCUSSION

Tuberous sclerosis complex disease is caused by loss-of-function variants in either the \textit{TSC}1 gene localized on the 9q34 region or in the \textit{TSC}2 gene located on the 16p13.3 region. The revised tuberous sclerosis complex diagnostic criteria published in 2013 by the International Tuberous Sclerosis Complex Consensus Group include the screening of \textit{TSC}1/\textit{TSC}2 genes, emphasizing the importance of genetic screening of \textit{TSC} genes in suspected patients.\textsuperscript{8} We have presented our genetic analysis results of 23 patients referred to our Genetic Diseases Diagnosis Center to be tested in terms of \textit{TSC}1/\textit{TSC}2 variants that we analyzed both with HTS and MLPA methods. We found a large deletion including partial regions of both \textit{TSC}2 and \textit{PKD}1 genes in twin sisters and defined 4 \textit{TSC}2 variants, one of them being a mosaic in-frame deletion.

The \textit{TSC}1 gene has 23 exons in its canonical transcript (NM_000368) and encodes the cell adhesion protein called hamartin, whereas the \textit{TSC}2 gene encoding for tuberin is a relatively large gene comprising 42 exons in its canonical transcript. Previous studies have shown that the variant frequency and diversity of the \textit{TSC}2 gene are higher than the \textit{TSC}1 gene. Furthermore, \textit{TSC}2 variants are associated with a more severe phenotype than those in \textit{TSC}1.\textsuperscript{28,29} In 8 out of 23 patients, we identified pathogenic/likely pathogenic \textit{TSC}2 variants. However,
| Patient No. | Age/Gender | Genetic Screening Results                                                                 | Clinical/Phenotypic Information                                                                 | Diagnosis According to TvSC Updated Criteria Without Genetic Testing | Diagnosis According to TSC Updated Criteria After Genetic Testing | Family History of TSC |
|------------|------------|------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|---------------------------------------------------------------------|------------------------------------------------------------------|------------------------|
| P 1        | 29/F       | Heterozygous c.4253dupC(p. Gln1419ThrfsTer105) in TSC2                                     | Bilateral renal multiple angiomyolipomas Thyroid nodules and bone marrow involvement         | NA                                                                  | DD                                                              | No                     |
| P 2        | 11/M       | Heterozygous c.138+2T>A in TSC2                                                           | T2Flair increased signal intensity Maxillary retention cysts                                 | DD                                                                  | DD                                                              | Yes, son of patient 3  |
| P 3        | 53/F       | Mosaic c.1000_1002delGTG (p.Val334del) in TSC2                                             | Cranial cysts compatible with TSC                                                            | DD                                                                  | DD                                                              | Yes, mother of patient 2 |
| P 4        | 4/F        | Heterozygous c.5192dupA (p.Asn1731LysfsTer44) in TSC2                                     | T2Flair increased signal intensity Multiple hyperechogenic nodules in liver                    | DD                                                                  | DD                                                              | No                     |
| P 5        | 21/F       | Heterozygous deletion of TSC2 33-42nd exons and PKD1-30/PKD1-40 gene regions (TSC2/PKD1 contiguous deletion syndrome) | Cranial tubers Polycystic kidney disease Polycystic kidney disease                         | DD                                                                  | DD                                                              | Yes, mother of patient 6 |
| P 6        | 1/M        | None                                                                                     | Cranial tubers Hypomelanocytic macules                                                      | DD                                                                  | DD                                                              | Yes, son of patient 5  |
| P 7        | 9/F        | None                                                                                     | Cranial tubers Resistent epilepsy and learning disability                                   | DD                                                                  | DD                                                              | Yes, twin sister of patient 8                                   |
| P 8        | 9/F        | None                                                                                     | Cranial tubers ADHD Epileptic seizures until 4 years old                                  | DD                                                                  | DD                                                              | Yes, twin sister of patient 7                                   |
| P 9        | 14/F       | None                                                                                     | Gray matter heterotopia in the left cerebral hemisphere                                    | NA                                                                  | NA                                                              | No                     |
| P 10       | 10/M       | None                                                                                     | Epilepsy                                                                                    | NA                                                                  | NA                                                              | No                     |
| P 11       | 12/M       | None                                                                                     | Cortical dysplasia in brain MRI Epilepsy                                                    | NA                                                                  | NA                                                              | No                     |
we did not find any pathogenic/likely pathogenic variants of the TSC1 gene.

Mosaic variants of the TSC1 or TSC2 gene in TSC cases with various mutant allele frequencies have been reported in index cases and asymptomatic or mildly symptomatic parents of TSC cases. In 1 of our cases, the in-frame deletion in the TSC2 gene was evaluated as a mosaic with 27% mutant allele frequency according to the results of HTS. In our patient’s brain magnetic resonance images, besides cysts compatible with TSC, there were angiolipomas and cysts in both kidneys, and she had a history of epileptic seizures. Thus, along with other TSC patients found to have in-frame deletions.

### TABLE 3. High-Throughput Sequencing and Multiplex Ligation-Dependent Probe Amplification Screening Results and Clinical Features of the Patients (Continued)

| Patient No. | Age/Gender | Genetic Screening Results | Clinical/Phenotypic Information | Diagnosis According to tvSC Updated Criteria Without Genetic Testing | Diagnosis According to TSC Updated Criteria After Genetic Testing | Family History of TSC |
|-------------|------------|---------------------------|--------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|------------------------|
| P 12        | 1/M        | None                      | Epileptic seizures             | NA                                                            | NA                                                            | No                     |
| P 13        | 15/M       | None                      | Bilateral tubers in brain MRI  | DD                                                            | DD                                                            | No                     |
|             |            |                           | Ewing Sarcoma                  |                                                               |                                                               |                        |
|             |            |                           | Facial angiofibroma             |                                                               |                                                               |                        |
|             |            |                           | Hypopigmented macules          |                                                               |                                                               |                        |
| P 14        | 5/F        | None                      | Cranial hamartoma and cortical dysplasia | NA                                            | NA                                                            | No                     |
|             |            |                           | Overgrowth                      |                                                               |                                                               |                        |
|             |            |                           | Macrocephaly                    |                                                               |                                                               |                        |
|             |            |                           | Frontal bossing                 |                                                               |                                                               |                        |
|             |            |                           | Multiple cafe-au-lait macules   |                                                               |                                                               |                        |
| P 15        | 1/F        | None                      | Epileptic seizures             | NA                                                            | NA                                                            | No                     |
| P 16        | 1m/M       | None                      | Rhabdomyoma                     | NA                                                            | NA                                                            | Yes                    |
|             |            |                           | Brother with the clinical diagnosis of TSC (8 years old) |                                                               |                                                               |                        |
| P 17        | 1/M        | None                      | Hypopigmented macule (1X)       | NA                                                            | NA                                                            | No                     |
|             |            |                           | Rhabdomyoma in prenatal history |                                                               |                                                               |                        |
| P 18        | 1/F        | None                      | Hypopigmented macule (1X)       | NA                                                            | NA                                                            | No                     |
|             |            |                           | Epileptic seizures             |                                                               |                                                               |                        |
| P 19        | 5/F        | None                      | Epileptic seizures             | NA                                                            | NA                                                            |                        |
|             |            |                           | Hypotonia                       |                                                               |                                                               |                        |
|             |            |                           | Growth retardation              |                                                               |                                                               |                        |
| P 20        | 10/F       | None                      | Epilepsy                        | NA                                                            | NA                                                            | No                     |
|             |            |                           | Mental motor retardation        |                                                               |                                                               |                        |
|             |            |                           | Long face                       |                                                               |                                                               |                        |
|             |            |                           | Broad forehead                  |                                                               |                                                               |                        |
|             |            |                           | Flat philtrum                   |                                                               |                                                               |                        |
|             |            |                           | Dysplastic ears                 |                                                               |                                                               |                        |
|             |            |                           | Thin upper lip                  |                                                               |                                                               |                        |
| P 21        | 11/F       | None                      | Cranial tubers                  | DD                                                            | DD                                                            | No                     |
|             |            |                           | Subependymal nodule             |                                                               |                                                               |                        |
|             |            |                           | Multiple hypopigmented          |                                                               |                                                               |                        |
| P 22        | 11/F       | None                      | Cortical tubers in brain MRI    | DD                                                            | DD                                                            | Yes                    |
|             |            |                           | Rhabdomyoma                     |                                                               |                                                               |                        |
|             |            |                           | Familial history of TSC         |                                                               |                                                               |                        |
| P 23        | 10/F       | None                      | T2Flair increased signal intensity | NA                                            | NA                                                            | No                     |

F, female; M, male; m, months; ID, intellectual disability; NA, not available; DD, definite diagnosis; PD, possible diagnosis.
TABLE 4. Variants Defined in TSC2 Gene Via High-Throughput Sequencing Analysis

| TSC2 cDNA change (NM_000548.5) | Predicted Protein Change | Variant Type | CADD Score | MutationTaster | MaxEntScan | GeneSplicer | gnomAD | ACMG Classification |
|--------------------------------|--------------------------|--------------|------------|----------------|------------|-------------|--------|---------------------|
| c.138+2T>A                     | NA                       | Splice variant | 33         | Disease-Causing | Likely disrupted | NA |
| c.1000_1002delGTG              | p.(Val334del)            | In-frame deletion | 21         | Disease-Causing | NA | NA | 0% | Likely pathogenic (PM2, PP1) |
| c.4253dupC                     | p.(Gln1419ThrfsTer105)   | Frameshift duplication | 44         | Disease-Causing | NA | NA | 0% | Likely pathogenic (PM2, PM4, PM6) |
| c.5192dupA                     | p.(Asn1731LysfsTer44)    | Frameshift deletion | 34         | Disease-Causing | NA | NA | 0% | Pathogenic (PP1, PKS1, PP2) |

NA, not available.

in the previous studies,29,30 the compatible clinical features of our patient confirm the pathogenicity of this novel mosaic in-frame deletion.

Splicing variants are common in TSC genes, and there are 134 pathogenic/likely pathogenic splice site variants of the TSC2 gene reported in the ClinVar database. The c.138+2T>A splice variant of the TSC2 gene defined in our patient was inherited from his affected mother. Another single nucleotide change, c.138+2T>C, had been reported in patients with TSC in a previous report,35 but this variant was a novel variant neither reported in previous reports nor in public databases. The absence of this canonical splice site variation (PVS1 according to ACMG 2015) in public databases (PM2 according to ACMG2015) and the presence of the variant both in the affected mother and son supported the pathogenicity of this novel splice site variation in our study.

The PKD1 gene and the TSC2 gene are located nearby on the 16q chromosome in a tail-to-tail orientation. Variants and deletions of the PKD1 gene are associated with autosomal dominant polycystic kidney disease.32 In 1994, Brook-Carter et al. reported TSC patients with deletions encompassing TSC2 and PKD1 genes, who have severe polycystic kidney disease in infancy.32 The phenotype, resulting from the large deletions including the TSC2 and PKD1 genes, was then classified as TSC2/PKD1 contiguous gene syndrome (PKDTS, MIM#600273). In our patient cohort, we defined 2 patients with PKDTS who are twin sisters. Both of them had polycystic kidney disease. Previous studies reported that the deletion size does not correlate with clinical features.32 Unfortunately, it was impossible to compare genotype–phenotype correlation because we did not have any other patients except the twin sisters with PKDTS.

Tuberous sclerosis is a disease in which phenotypic diversity can be seen even among individuals carrying the same variant in the family, but in which penetrance is complete.3 In 2 of the familial patients in our cohort, the variants were inherited from affected mothers by their affected sons (patients 2 and 3; patients 5 and 6). The 2 other familial patients (patients 7 and 8) were twin sisters with TSC2/PKD1 contiguous deletion syndrome. All familial patients, even the twin sisters, had phenotypic discordance supporting the expressivity differences of TSC.

The pathogenic/likely pathogenic variant frequency was lower in this study, and there are some possible reasons. First of all, the patient group included not only the clinically diagnosed TSC patients but also the patients with signs and symptoms of TSC, listed in the HPO database. The variant definition rate of patients with a possible or definite diagnosis of TSC was 8/11, reflecting the importance of clinical criteria prior to genetic evaluation. Epileptic seizures were more common in the variant-negative patient group. Whereas idiopathic epilepsy may be seen in most TSC patients, there may be very different causative genes underlying epileptic seizures. The high penetrance of epilepsy in our variant-negative patient group supports the reports suggesting the need for a multi-gene HTS panel for clarifying the genetic background of idiopathic epilepsy rather than testing only the TSC1 and TSC2 genes.33 As a second restrictive point, the designed kit used in this study has a 20 bp intron padding. Studies indicate that the deep intronic variants may cause TSC, and it is possible that we might not define deep intronic mutations with the designed kit.33 The other possibility is that some low-level mosaic variants that can be seen in TSC34,35 may be missed even in the HTS analysis, detected with germline mutation-detection pipelines.

In conclusion, we reported 3 novel and 1 known TSC2 variants, including a mosaic in-frame deletion and a large deletion encompassing partial regions of both TSC2 and PKD1 genes, causing the PKDTS phenotype. Detection of mosaic variants can be challenging even in HTS analysis. Therefore, care should be taken that stringent quality parameters are being used in HTS analysis, keeping in mind the possibility of mosaic variants with low allelic fractions. We suggest that different clinical and phenotypic features of even the members of the same family, the novelty of most of the variants found, and the presence of a mosaic likely pathogenic variant even in the small-sized study population support the importance of a comprehensive approach in the genetic diagnosis of TSC. To our knowledge, this is the first study reporting both HTS and MLPA results of TSC1/TSC2 genes of the patients with signs and symptoms of TSC in the Turkish population.

Ethics Committee Approval: The Institutional Ethical Board approved this study with the decision number BAEK-2021/45.

Patient Consent for Publication: Written informed consent was obtained from the patients or the legal guardians of the patients younger than 18 years old.
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