Mutation analysis of TSC2 gene in 33 Turkish familial cases with tuberous sclerosis

Anıl Apak[1], Göknur Haliloğlu[1], Gülşen Köse[2], Engin Yılmaz[3], Banu Anlar[1], Sabiha Aysun[1]

Departments of [1]Pediatric Neurology, and [3]Medical Biology, Hacettepe University Faculty of Medicine and [2]Department of Pediatric Neurology, Social Security Children's Hospital, Ankara, Turkey

SUMMARY: Apak A, Haliloğlu G, Köse G, Yılmaz E, Anlar B, Aysun S. Mutation analysis of TSC2 gene in 33 Turkish familial cases with tuberous sclerosis. Turk J Pediatr 2003; 45: 1-5.

Tuberous sclerosis is an autosomal dominant multisystem disorder characterized by hamartomatous growths in different organs. Disease determining genes are localized to 9q34 (TSC1) and 16p13.3 (TSC2). Two-thirds of the cases are sporadic and result from new mutations. The aim of this study was to determine TSC2 gene mutations by Single Stranded Conformation Polymorphism (SSCP) analysis and direct sequencing in 33 familial cases with tuberous sclerosis who were followed up in the Pediatric Neurology Departments of Hacettepe University İhsan Doğramacı and Ankara Social Security Children's Hospitals.

Forty-one exons of TSC2 gene were amplified and subjected to SSCP, and sequence analysis was performed when an abnormal SSCP pattern was observed. As a result, six new mutations and nine gene polymorphisms were detected. The new mutations are G→T mutation in exon 20, 16bp deletion in exon 29, 18bp deletion in exon 40, 538 G→A mutation in exon 29, T→C mutation in exon 21 and G→A splice site mutation in exon 5.

Although further studies on larger groups are needed, these results do not indicate a common region or type of mutation in the Turkish population.

Key words: tuberous sclerosis, TSC2, mutation, single stranded conformation polymorphism.

Tuberous sclerosis (TS), or Bourneville's disease, is an autosomal dominant, multisystemic, neurocutaneous disease characterized by the growth of tumor-like hamartomas in different organs. Its incidence is reported to vary between 1/6,000-1/20,000 births[1]. About two-thirds of the cases are sporadic without a known family history of the disorder[1]. Hypopigmented macules, facial angiofibromas, cardiac rhabdomyomas, cortical tubers and subependymal nodules are among the most common clinical findings[2]. The central nervous system is the most severely affected system and patients present with seizures, mental retardation and autistic features[3]. In the Tuberous Sclerosis Consensus Conference 1998, clinical diagnostic criteria of TS were revised and a new classification based on major and minor symptoms[4] was established (Table I). Instead of multiple lesions of the same type in the same organ system, two or more distinct types of lesions are required for the definitive diagnosis of TS[4].

Two disease-determining genes were defined for TSC1 and TSC2, on chromosomes 9q34 and 16p13, respectively[5-7]. TSC1 gene has 23 exons and its protein product is hamartin[6,7]. TSC2 gene has 41 exons in the coding region and a leader exon with a transcript existing as multiple isoforms; its protein product is tuberin[6,7]. Both hamartin and tuberin are tumor-suppressor proteins involved in cellular growth and differentiation[6,7]. In some series each locus is defined to account for 50% of the familial cases, while other laboratories indicate that only 10%-20% of patients with TS have TSC1 mutations[6-10].

The aim of this study was to define TSC2 gene mutations by Single Stranded Conformation Polymorphism (SSCP) and direct sequence analysis in 33 familial cases of TS who were followed up in the Pediatric Neurology Department of Hacettepe University İhsan Doğramacı and Ankara Social Security Children's Hospitals.

Table I. Revised Diagnostic Criteria for Tuberous Sclerosis Complex*

Major Features
- Facial angiofibromas or forehead plaque
- Nontraumatic ungual or periungual fibroma
- Hypomelanotic macules (three or more)
- Shagreen patch (connective tissue nevus)
- Multiple retinal nodular hamartomas
- Cortical tuber
- Subependymal nodule
- Subependymal giant cell astrocytoma
- Cardiac rhabdomyoma, single or multiple
- Lymphangiomyomatosis
- Renal angiomyolipoma

Minor Features
- Multiple, randomly distributed pits in dental enamel
- Hamartomatous rectal polyps
- Bone cysts
- Cerebral white matter radial migration lines
- Gingival fibromas
- Nonrenal hamartoma
- Retinal achromic patch
- Confetti skin lesions
- Multiple renal cysts

Definite TS: two major features or one major feature plus two minor features.
Probable TS: One major plus one minor feature.
Possible TS: One major feature or two or more minor features.

* Tuberous Sclerosis Complex Consensus Conference: Revised Clinical Diagnostic Criteria J Child Neurol 1998; 13: 624-628.

Material and Methods

Thirty-three children diagnosed with TS according to the established criteria[4] who had at least one other affected family member were studied for TSC2 mutations.

Genomic DNA was extracted from peripheral blood. Mutational analysis for TSC2 gene through exons 1 to 41 was done using primer sets described before[11]. After standard polymerase chain reaction (PCR) amplification (200 ng of genomic DNA was amplified in a volume of 10 µl for 30 cycles, consisting of 1 min of denaturing at 94°C, 30 sec-1 min of annealing at 55-62°C, and 30 sec-1 min of elongation at 72°C), mutations were searched by SSCP analysis. The samples were diluted in 1:1 ratio in buffer containing 0.05% sodium dodecyl sulfate (SDS), 2 mM disodium ethylenediamine tetraacetate (EDTA), 47.5% formamide, 0.05% bromphenol blue, and 0.05% xylene cyanol. The diluted products were denatured at 95°C for 5 min and analyzed on nondenaturing gels containing 6-8% polyacrylamide and 8% glycerol. Each exon of the TSC2 gene was marked with P^{32} and, after reamplification by PCR, the gels were electrophoresed at 6-8 W for 14-16 hours, dried and exposed to Kodak film. For each exon, mobility of DNA was compared with positive, negative and nondenaturing samples. When an abnormal pattern such as a mobility shift in SSCP analysis was observed, direct sequence analysis was performed using exonucleases (EXO-1 and SAP: Shrimp Alkaline Phosphatase). The sequencing reaction was performed for 26 cycles of 1 min denaturing at 95°C, 1 min annealing at 55-62°C, and 1 min elongation at 72°C, followed by termination of the reaction with stop solution. The samples were loaded on a denaturing polyacrylamide gel, dried and exposed to Kodak film.

Results

We identified six previously undescribed mutations (18.1%) and nine gene polymorphisms (27.2%) in 33 familial cases with tuberous sclerosis. These new mutations were G→T mutation in exon 20, 16 bp deletion in exon 29, 18 bp deletion in exon 40, 538 G→A mutation in exon 29, T→C mutation in exon 21 and G→A splice-site mutation in exon 5. Polymorphisms included exon 5 polymorphism (2 patients), exon 40 polymorphism (4 patients), exon 33 polymorphism (1 patient), exon 27 polymorphism (1 patient) and exon 14 and 40 polymorphism (1 patient). All patients with new mutations had both skin and central nervous system involvement and no particular features, except the patient with G→T mutation in exon 20 who manifested severe autistic features.

The clinical findings in patients with new mutations are documented in Table II.

Discussion

Tuberous sclerosis is an autosomal dominant disease with growth of tumor-like hamartomas in different organs. In 1987, a linkage to chromosome 9 was found in 19 families with tuberous sclerosis[5]. TSC1 linkage was found to represent about 30% of the cases with tuberous sclerosis and, because of disease heterogeneity, a second locus on chromosome 16p13, at the locus of autosomal dominant polycystic kidney disease, was defined and named TSC2[7]. The TSC2 gene protein product tuberin is

Table II. Clinical Findings of Patients with New Mutations in TSC2 Gene

New mutation	Age, sex	Symptom	CNS involvement	Skin involvement
G→T mutation exon 20, exon 5 polymorphism	9 y, M	Focal seizure at 2 months, motor and mental retardation severe autistic features	Subependymal calcification	Adenoma sebaceum, Hypopigmented macule
16bp deletion in exon 29	11 y, M	Generalized tonic clonic seizures at 1 yr	Subependymal calcification	Adenoma sebaceum, Hypopigmented macule
18bp deletion in exon 40	6 y, F	Febrile and afebrile seizures after age 2	Subependymal calcification	Hypopigmented macule, Shagreen patch
538 G→A mutation exon 29	1.5 y, F	Infantile spasms at the age of 3 months	Subependymal calcification	Hypopigmented macule
T→C missense mutation exon 21	3 y, F	Seizures at the age of 1 year	Subependymal calcification	Hypopigmented macule
G→A splice-site mutation exon 5	4 y, F	Infantile spasms at the age of 6 months	Subependymal calcification	Hypopigmented macule

CNS: central nervous system.

synthesized in different organs, mainly brain and kidney, and also in hamartomatous lesions. The structure of tuberin is similar to Rap1 oncogene activator protein[12]. Determination of mutations in tumoral growths in TSC2 patients indicate that TSC2 gene functions as a tumor-suppressor gene[12,13]. Both hamartin and tuberin are necessary for cellular growth and differentiation[6,7,12,13]. Loss of heterozygosity (LOH) in 16p locus was found in tumoral tissues such as cardiac rhabdomyoma, renal angiomyolipoma, subependymal cortical tuber, retinal hamartomas and giant cell astrocytoma[14,17].

Therefore, TS belongs to the family of tumor-suppressor gene syndromes, and affected individuals have at least one inactivating mutation in either TSC1 or TSC2 genes in all germ-line and somatic cells. Many patients have LOH in either TSC1 or TSC2, which is a finding consistent with the two-hit hypothesis[15-17].

TSC2 gene has 41 exons in the coding region and a leader exon. Only 2-3% of TSC2 gene mutations are large deletions; subtle mutations are more common[8]. Mutations are distributed throughout the coding sequence without a specific hot spot. Since both TSC1 and TSC2 genes have a high number of exons, mutations can occur anywhere within the gene, which makes the genetic analysis rather difficult and time consuming. TSC1 and TSC2 genes represent approximately 50% of the familial cases; however, according to Dabora et al.[10] these studies are all limited because the families studied usually had two affected individuals without extension of TS to multigenerations, and because small numbers of families were studied. The exact proportion of patients with TSC1 and TSC2 mutations varies also according to the method, but TSC1 mutations represent 10%-20% of all mutations and TSC2 mutations seem to be more frequent in both familial and sporadic cases[10]. Also, about 17% of TS cases remain where a mutation can not be defined[10]. This suggests the possibility of an unidentified third locus. Other reasons for relatively few TSC1 and TSC2 gene mutations being defined despite intensive molecular analysis and efforts include: the low frequency or absence of mutant allele in peripheral blood leukocytes, the absence of a hot spot within TSC1 and TSC2 genes which have 23 and 41 exons respectively, factors regulating gene function and posttranscriptional modifications, lack of a universal routinely available genetic test, environmental factors, and failure to detect mosaicism. Mosaicism is defined as an entity in which a fraction of germ-line and somatic cells contain a mutation or chromosomal abnormality[14,17]. Mosaicism for TSC2 gene mutations with milder clinical findings in cases who had TS and polycystic kidney disease were

also defined[14,15]. As in diseases like TS where spontaneous mutations occur, mosaicism is a serious potential problem in the genetic diagnosis and counseling[1,4]. For example, mutation was present in only one-third of leukocytes and was undetectable in a sample of buccal mucosa DNA in a patient with TSC1[14].

Although definitive diagnosis depends on criteria based on clinical, radiological and histopathological findings, great effort has been spent to define mutations and genotype-phenotype correlations after the determination of TSC1 and TSC2 genes[5-7]. These attempts at correlating genotype and phenotype rendered controversial results[8-10,18]. Beauchamp et al.[8] examined TSC2 gene mutations in 20 familial and 20 sporadic cases and identified 21 mutations representing 50% of familial and 55% of sporadic cases. They suggested that at least 50% of sporadic cases arise from mutations in TSC2, and there were no clinical differences nor genotype-phenotype correlation between TSC1 and TSC2 patients.

Niida et al.[9] examined TSC1 and TSC2 gene mutations in 40 familial and 86 sporadic patients by SSCP analysis followed by direct sequencing, and identified mutations in 59% of the cases. Sixteen of these mutations were TSC1 (5 sporadic, 11 familial), and 58 of these mutations (42 sporadic, 16 familial) were TSC2. There was again no correlation between genetic analysis and clinical presentation in either group[9].

In a series from Japan, 23 new mutations were defined in 38 patients with TS, and although patients with TSC2 mutations tended to have relatively severe mental retardation, a genotype-phenotype correlation was not estaslished[19].

Dabora et al.[10] studied 224 patients with TS by denaturing high performance liquid chromatography (DHPLC), PCR and quantitative PCR, and found a total of 186 (83%) mutations, including small TSC2 (n. 138), large TSC2 (n:20) and small TSC1 (n: 28) mutations. Missense and frame/deletion mutations were only observed in TSC2 patients. The same study also indicated that sporadic patients with TSC1 mutations have milder disease compared to patients with TSC2 mutations, including lower frequency of seizures, moderate-to-severe mental retardation, fewer subependymal nodules and tubers, less severe kidney involvement and facial angiofibroma and no retinal hamartoma[10].

Most studies demonstrate a failure to predict clinical status based on genetic abnormality. Considering that many of the clinical features are age-dependent, studies involving phenotype should contain a sufficient number of patients from different age subgroups for statistical analysis. We studied TSC2 gene mutations because the gene has a higher number of exons compared to TSC1, the analysis is rather time consuming, and mutations are more frequent in familial and severe cases. Among 33 familial TS patients, six new mutations and nine gene polymorphisms for TSC2 were defined. The patient with G→T mutation in exon 20 exhibited severe autistic features in addition to skin involvement. In the other four patients with new mutations, there was skin and central nervous system involvement, but no specific clinical feature. Future studies will include TSC1 mutations in the same patient group and also in sporadic cases.

Acknowledgements

Dr. Apak was supported by a program from TÜBİTAK.

This work was presented in part at the 2nd National Child Neurology Congress, May 2000, Ankara.

The authors thank Dr. Vijaya Ramesh and Dr. Yo Niida from the Molecular Neurogenetics Unit, Massachusetts General Hospital, Charlestown, MA, for their support in molecular genetic analysis.

The DNA samples were isolated and stored in the Repository of TÜBİTAK DNA/Cell BANK.

REFERENCES

1. Osborne JP, Fryer A, Webb D. Epidemiology of tuberous sclerosis Ann NY Acad 1995; 615: 125-127.
2. Roach ES, Delgado MR. Tuberous sclerosis. Dermatol Clin 1995; 13: 151-161.
3. Curatolo P, Cortesi R, Chiron C, Jambaque I, Dulac O. Neuropsychiatric aspects of tuberous sclerosis. Ann NY Acad Sci 1991; 615: 8-16.
4. Roach ES, Gomez MR, Northrup H. Tuberous Sclerosis Complex Consensus Conference: Revised Clinical Diagnostic Criteria. J Child Neurol 1998; 13: 624-628.
5. Fryer AE, Chalmers AH, Connor JM, et al. Evidence that the gene for tuberous sclerosis is on chromosome 9. Lancet 1987; 1: 659-661.
6. Gomez MR. History of the tuberous sclerosis complex. Brain Dev 1995; 17 (Suppl): 55-71.
7. Kandt RS, Haines JL, Smith M, et al. Linkage of an important gene locus for tuberous sclerosis to a chromosome 16 marker for polycystic kidney disease. Nat Genet 1992; 2: 37-41.

8. Beauchamp R, Banwell A, McNmara P, et al. Exon scanning of the entire TSC2 gene for germline mutations in 40 unrelated patients with tuberous sclerosis. Hum Mutat 1998; 12: 408-416.
9. Niida Y, Lawrence-Smith N, Banwell A, et al. Analysis of both TSC1 and TSC2 for germline mutations in 126 unrelated patients with tuberous sclerosis. Hum Mutat 1999; 14: 412-422.
10. Dabora SL, Jozwiak S, Franz DL, et al. Mutational analysis in a cohort of 224 tuberous sclerosis patients indicates increased severity of TSC2, compared with TSC1 disease in multiple organs. Am J Hum Genet 2001; 68: 64-80.
11. Au K-S, Rodriguez JA, Finch JL, et al. Germline mutational analysis of the TSC2 gene in 90 tuberous sclerosis patients. Am J Hum Genet 1998; 62: 286-294.
12. Cheadle JP, Reeve MP, Sampson JR, Kwiatkowski DJ. Molecular genetic advances in tuberous sclerosis. Hum Genet 2000; 107: 97-114.
13. Sepp T, Yates JN, Green AJ. Loss of heterozygosity in tuberous sclerosis hamartomas. J Med Genet 1996; 33: 962-964.
14. Kwiatkowska J, Wigowska-Sowiniska J, Napierala D, Slomski R, Kwiatkowski DJ. Mosaicism in tuberous sclerosis as a potential cause of the failure of molecular diagnosis. N Engl J Med 1999; 340: 703-707.
15. Sampson JR, Maheshwar MM, Aspinwall R, et al. Renal cystic disease in tuberous sclerosis: role of the polycystic disease 1 gene. Am J Hum Genet 1997; 61: 843-851.
16. Henske EP, Scheithauer BW, Short MP, et al. Allelic loss is frequent in tuberous sclerosis kidney lesions but rare in brain lesions. Am J Hum Genet 1996; 59: 400-406.
17. Green AJ, Smith M, Yates JR. Loss of heterozygosity on chromosome 16p13.3 in hamartomas from tuberous sclerosis patients. Nat Genet 1994; 6: 193-196.
18. van Slegtenhorst M, Verhoef S, Tempelaars A, et al. Mutational spectrum of the TSC1 gene in a cohort of 225 tuberous sclerosis patients: no evidence for genotype-phenotype correlation. J Med Genet 1999; 36: 285-289.
19. Zhag H, Nanba E, Yamamoto T, et al. Mutational analysis of TSC1 and TSC2 genes in Japanese patients with tuberous sclerosis complex. J Hum Genet 1999; 44: 391-396.