Mutational analysis of TSC1 and TSC2 genes in Tuberous Sclerosis Complex patients from Greece

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Tuberous sclerosis complex (TSC) is a rare autosomal dominant disorder causing benign tumors in the brain and other vital organs. The genes implicated in disease development are TSC1 and TSC2. Here, we have performed mutational analysis followed by a genotype-phenotype correlation study based on the clinical characteristics of the affected individuals. Twenty unrelated probands or families from Greece have been analyzed, of whom 13 had definite TSC, whereas another 7 had a possible TSC diagnosis. Using direct sequencing, we have identified pathogenic mutations in 13 patients/families (6 in TSC1 and 7 in TSC2), 5 of which were novel. The mutation identification rate for patients with definite TSC was 85%, but only 29% for the ones with a possible TSC diagnosis. Multiplex ligation-dependent probe amplification (MLPA) did not reveal any genomic rearrangements in TSC1 and TSC2 in the samples with no mutations identified. In general, TSC2 disease was more severe than TSC1, with more subependymal giant cell astrocytomas and angiomyolipomas, higher incidence of pharmacoresistant epileptic seizures, and more severe neuropsychiatric disorders. To our knowledge, this is the first comprehensive TSC1 and TSC2 mutational analysis carried out in TSC patients in Greece.
Identification and characterization of mutations. In the present study, we performed mutational analysis in the coding exons and intron/exon junctions of both TSC1 and TSC2 in a total of twenty patients/families. TSC mutation screening was carried out in the probands, but whenever possible, the presence of pathogenic mutations was investigated in parental DNAs and in other family members as well. In total, thirteen sequence variants was investigated in parental DNAs and in other family members as well. In total, thirteen sequence variants were identified in two patients/families.

Table 1. Clinical characteristics of TSC patients from Greece in the present study. CD: Cortical dysplasias; SEG: Subependyomal giant cell astrocytomas; AML: Renal angiomylipomas; LAM: Lymphangioleiomyomatosis; MRH: Multiple retinal hamartomas; AF: Angiofibromas; SP: Shagreen patches; LD: Learning disabilities; MR: Mental retardation; ASD: Autism spectrum disorder; na: Information not available. * Based on molecular diagnosis in the present study.

| Patient | Sex | Gene* | TSC diagnostic status | Neurological findings | Renal findings | Pulmonary features | Ophthalmologic features | Dermatological findings | TAND |
|---------|-----|-------|-----------------------|-----------------------|----------------|-------------------|------------------------|------------------------|------|
| 1       | F   | TSC2  | Definite              | —                     | +              | +                 | —                      | —                      | —    |
| 2       | M   | TSC2  | Definite              | +                     | +              | +                 | —                      | —                      | —    |
| 3       | M   | TSC2  | Definite              | —                     | —              | —                 | —                      | —                      | —    |
| 4       | F   | TSC1  | Definite              | +                     | +              | +                 | —                      | —                      | —    |
| 5       | F   | TSC2  | Definite              | +                     | +              | +                 | —                      | —                      | —    |
| 6       | M   | TSC1  | Definite              | +                     | +              | +                 | —                      | —                      | —    |
| 7       | M   | TSC2  | Possible              | +                     | +              | +                 | —                      | —                      | —    |
| 8       | F   | TSC2  | Definite              | +                     | +              | +                 | —                      | —                      | —    |
| 9       | M   | TSC2  | Definite              | —                     | +              | +                 | —                      | —                      | —    |
| 10      | F   | TSC1  | Definite              | —                     | +              | +                 | —                      | —                      | —    |
| 11      | F   | TSC2  | Definite              | +                     | +              | +                 | —                      | —                      | —    |
| 12      | F   | Possible | +     | +              | +                 | —                      | —                      | —                      | —    |
| 13      | F   | Possible | +     | +              | +                 | —                      | —                      | —                      | —    |
| 14      | F   | TSC1  | Definite              | +                     | —              | +                 | —                      | —                      | —    |
| 15      | F   | TSC1  | Definite              | —                     | —              | +                 | —                      | —                      | —    |
| 16      | M   | Possible | +     | +              | +                 | —                      | —                      | —                      | —    |
| 17      | F   | Possible | +     | +              | +                 | —                      | —                      | —                      | —    |
| 18      | M   | Possible | +     | +              | +                 | —                      | —                      | —                      | —    |
| 19      | M   | TSC2  | Definite              | +                     | —              | +                 | —                      | —                      | —    |
| 20      | F   | TSC1  | Possible              | +                     | —              | +                 | —                      | —                      | —    |
The mutations presented in this work are c.1681_1700del20 (p.Ser561Glyfs*20) and c.2263C>T (p.Gln755*) in TSC1; c.648+1G>T, c.826delA (p.Met276Cysfs*17) and c.4942A>T (p.Ile1648Phe) in TSC2. No recurrent mutations were identified in either gene.

In more details, six disease-causing mutations were identified in TSC1 (46%), and seven in TSC2 (54%). TSC1 sequence variants included 3 nonsense mutations producing premature termination codons; 2 deletions, which caused frameshifts also resulting in the truncation of the produced protein; and 1 missense mutation. Three of these were familial mutations, and another three were de novo, while four out of the six had been previously reported in LOVD (Leiden Open Variation Database, http://chromium.lovd.nl/LOVD2/TSC/home.php).

Four of those had been reported previously, whereas 3 were novel (Table 2). The mutations identified by Sanger sequencing were not clustered on a particular exon in either of the genes, whereas no Copy Number Variants were detected in TSC1 or TSC2 using MLPA analysis. Finally, only one out of the four TSC families identified here presented with multiple (>2) members affected by TSC1 disease. This family is of interest as the affected individuals present with significant phenotypic differences in clinical characteristics (Fig. 1), suggesting that additional factors interfere with development of the disease phenotype.

### Table 2. TSC1 and TSC2 disease-causing mutations in TSC patients from Greece.

| Patient | Gene | Exon | Variant | Codon change | Mutation type | Inheritance | LOVD ID | ClinVar ID |
|---------|------|------|---------|--------------|---------------|-------------|---------|------------|
| 1 | TSC1 | 8 | c.737G>A | p.Arg246Lys | Missense | De novo | TSC1_00043 | 49094 |
| 6 | TSC1 | 9 | c.891C>T | p.Gln301* | Nonsense | Familial | TSC1_00056 | 49119 |
| 10 | TSC1 | 15 | c.1681_1700del20 | p.Ser561Glyfs*20 | Deletion | Familial | — | — |
| 20 | TSC1 | 18 | c.2249G>A | p.Trp750* | Nonsense | De novo | TSC1_00228 | 48922 |
| 14 | TSC1 | 18 | c.2263C>T | p.Gln755* | Nonsense | Familial | — | — |
| 7 | TSC2 | 21 | c.2701_2702delAG | p.Arg901Alafs*2 | Deletion | De novo | TSC1_00779 | — |
| 19 | TSC2 | 6 | c.648+1G>T | — | Splice-site | De novo | — | — |
| 1 | TSC2 | 8 | c.826delA | p.Met276Cysfs*17 | Deletion | De novo | — | — |
| 2 | TSC2 | 19 | c.2206_2210dup | p.Cys738Leufs*35 | Insertion | De novo | TSC2_00443 | 49600 |
| 8 | TSC2 | 20 | c.2221-2A>G | — | Splice-site | De novo | TSC2_00446 | 49815 |
| 9 | TSC2 | 23 | c.2713C>T | p.Arg905Trp | Missense | De novo | TSC2_00110 | 12404 |
| 5 | TSC2 | 37 | c.4942A>T | p.Ile1648Phe | Missense | De novo | — | — |
| 11 | TSC2 | 39 | c.5160+5G>T | — | Splice-site | Familial | TSC2_00651 | 49430 |

Figure 1. Family no 10: Mutation c.1681_1700del20 leads to different clinical characteristics in the affected family members.

Characterization of the additional variants identified. In this study, apart from the reported mutations with obvious or inferred pathogenic activity, additional variants have been identified. In total, 25 additional variants have been detected, from which 9 were found in TSC1 and 16 in TSC2, whereas 4 among them have not been reported previously (1 at the TSC1 locus and 3 at TSC2). These additional variants fell into two groups: (1) TSC1 and TSC2 variants co-existing with a TSC-causing mutation already detected in the bearers (Tables 3); and (2) TSC1 and TSC2 variants in patients with no TSC mutation identified (NMI) (Table 4).

In total, 17 out of the 25 additional variants identified were intronic. Since these could possibly have pathogenic activity due to their possible involvement in alternative splicing, we have screened LOVD and ClinVar for all additional variants, and found that in a significant percentage they have been characterized as benign or likely benign (Tables 3 and 4). As a conclusion, it is likely that most of the additional variants identified in this work that are also present in LOVD and/or ClinVar, are polymorphisms with no apparent clinical significance.
Familial vs de novo TSC mutations. A significantly higher percentage of patients with de novo TSC mutations (69%) were identified compared to familial cases (31%). Of the 4 familial mutations reported here, 3 (75%) were detected in TSC1 and 1 (25%) in TSC2. In the three TSC1 families (6, 10 and 14), the pathogenic variant was

| Patient | Gene | Intron/Exon | Variant | Codon change | Variation Type | LOVD* | ClinVar** | Our data*** |
|---------|------|-------------|---------|--------------|---------------|-------|-----------|-------------|
| 12      | TSC1 | exon 15     | c.1439–21delT | Intronic      | -- -- -- -- -- |       |           |             |
| 17,18   |      | exon 19     | c.2502 + 51 A > G | Intronic      | 9 2 ak nkp 1 np 2 |
| 13      | TSC2 | exon 10     | c.976–63 G > A | Intronic      | 22 4 ak nkp 1 np 1 |
| 13,16   |      | exon 15     | c.1600–14G > T | Intronic      | 62 3 ak nkp 6 b/lb 2 |
| 17      |      | exon 22     | c.2546–31G > A | Intronic      | 1 1 ak npn 2 b 1 |
| 13,16   |      | exon 22     | c.2546–12C > T | Intronic      | 18 3 ak nkp 5 b 2 |
| 13,16   |      | exon 22     | c.520T > C   | Silent        | 34 3 + nkp 8 b/lb 2 |
| 13,16   |      | exon 22     | c.2639+44C > G | Intronic      | 11 1 ak npk 2 b 2 |
| 12      |      | exon 31     | c.3815+15G > A | Intronic      | 14 2 ak nkp 4 b/lb 1 |
| 13,16   |      | exon 40     | c.5161–10A > C | Intronic      | 91 2 + nkp 5 b/lb 2 |
| 13      |      | exon 41     | c.5260–49G > T | Intronic      | 25 9 ak nkp 2 b 1 |
| 13      |      | exon 41     | c.5260–25G > C | Intronic      | 5 1 ak nkp 2 b 1 |
| 13,16   |      | exon 41     | c.5397G > C   | p.Ser1799Ser  | Silent        | 91 12 + nkp 6 b/lb 2 |

Table 3. TSC1 and TSC2 additional variants in TSC patients from Greece with a pathogenic mutation identified. *I: Number of patients with the specific variant identified; II: Number of patients with a pathogenic variant identified; III: Found (+) in non-affected individuals, nk: not known; IV: Clinical significance suggested, nkp: no known pathogenicity. **I: Number of independent submissions in the database; II: Clinical significance suggested, b: benign, lb: likely benign. ***Number of times present in our database.

Table 4. TSC1 and TSC2 additional variants in TSC patients from Greece with no mutation identified (NMI). *I: Number of patients with the specific variant identified; II: Number of patients with a pathogenic variant identified; III: Found (+) in non-affected individuals, nk: not known; IV: Clinical significance suggested, nkp: no known pathogenicity, np: not provided. **I: Number of independent submissions in the database; II: Clinical significance suggested, b: benign, lb: likely benign, np: not provided. ***Number of times present in our database.

Genotype-Phenotype Correlations. Definite vs possible TSC cases. By definition, possible TSC patients present few symptoms, and very likely, a milder disease expression. Indeed, this was the case with our study, where among patients with possible TSC, displaying a generally milder disease phenotype, only 2 were found to bear a disease-causing mutation and this was detected in the TSC1 gene.

TSC1 vs TSC2 disease. Generally, definite TSC2 patients presented with a more severe phenotype compared to definite TSC1 patients. While cortical dysplasias were often seen in both TSC1 and TSC2 disease (TSC1: 5/6 or 83%; TSC2: 5/7 or 71%), development of SEGAs was a characteristic of TSC2 probands only (TSC1: 1/5 or 20%; TSC2: 5/7 or 71%). Moreover, epileptic seizures were more severe in TSC2 patients, and this was followed by more pronounced TAND characteristics. Additionally, multiple retinal hamartomas and a single case of LAM were observed in TSC2 patients only, whereas AML were present in both TSC1 and TSC2 patients in rather similar percentages (TSC1: 2/6 or 33%; TSC2: 3/7 or 43%).

Familial vs de novo TSC mutations. A significantly higher percentage of patients with de novo TSC mutations (69%) were identified compared to familial cases (31%). Of the 4 familial mutations reported here, 3 (75%) were detected in TSC1 and 1 (25%) in TSC2. In the three TSC1 families (6, 10 and 14), the pathogenic variant was
The 4 affected individuals from the latter family display a different set of clinical characteristics: fibroadenomas plus SEGA with no epilepsy, SEGA with uncontrollable seizures, severe mental retardation, and autism, all induced by the same TSC1 mutation (Fig. 1).

On the contrary, in TSC2 family 11, the mutation was transmitted by the mother, and it was a splice-site mutation. In this family, in order to determine whether the c.5160+5G>T variant in intron 39 could be the cause of a splicing error, RT-PCR was performed on peripheral blood lymphocyte RNA obtained from the proband. Sequencing of the RT-PCR product revealed that this single nucleotide substitution was enough to induce skipping of TSC2 exon 39 (Fig. 2).

Among the 9 de novo cases, 3 TSC1 (33%) and 6 TSC2 (67%) mutations were found. In total, we have identified 4 frameshift, 3 nonsense, 3 missense, and 3 splice-site mutations.

### Prediction of structural consequences of TSC1 and TSC2 missense mutations

In order to assess possible pathogenicity of the Ile1648Phe (I1648F) TSC2 missense variant, which is presented in this work for the first time, we compared 3D-models of Ile1648Phe TSC2 variant versus wild-type, but also of p.Arg246Lys (R246K) TSC1 missense variant versus wild-type, as an evaluation of our prediction.

To investigate the effect of the TSC1 p.Arg246Lys missense mutation at the protein level, we first produced 3D-models of the core domain of wt hTSC1 protein and of its R246K variant, as described in Material & Methods. As shown in Fig. 3, the 3D-model of the TSC1 R246K variant is very similar to that of the wt protein. In addition, the 246 Arg/Lys side chains are involved in intra-molecular interactions in both models (Fig. 3). It is therefore unlikely that this sequence change affects either the structure or the interactions of the TSC1 protein. These observations are in line with data in the literature which show that the Arg246Lys substitution does not affect TSC1 function, and suggest that the effect of the TSC1 p.Arg246Lys mutation is rather a result of alternative splicing.

On the contrary, as shown by mapping of the Ile1648Phe change on the 3D-model of the catalytic domain of TSC2 produced as described in Materials & Methods section (Fig. 4), substitution of the Ile residue at position 1648 by the much bulkier Phe residue, is anticipated to disrupt the structural integrity of this TSC2 domain due to steric hindrance with hydrophobic residues of the region (shown in grey sticks in Fig. 4). Therefore, it is most likely that the TSC2 p.Ile1648Phe mutation, by disrupting the structure of the catalytic domain of the TSC2 protein, impedes its GAP activity.
Probands/families with no mutation identified (NMI). In this work, in 7/20 probands/families, pathogenic mutations could not be identified. All 7 cases were de novo, with relatively mild symptoms of the disease. Nevertheless, since NMI refers to definite TSC patients and given the fact that here only 2 out of the 7 were definite TSC cases, with the remaining 5 being characterized as possible TSC, only these 2 are clear NMI, whereas the rest could be alternatively suffering from a disease other than TSC.

Generally, in NMI cases, pathogenic mutations could have been missed mainly because (a) they are found in genomic areas of TSC1 or TSC2 that are not covered during genetic analysis; or (b) the individuals are mosaics with just a small percentage of cells with a mutated TSC1 or TSC2.

In studies similar to the present one, where genetic testing is carried out in a diagnostic setting, usually, analysis of promoter regions, 5′- and 3′-UTRs and deep intronic areas of TSC1 and TSC2 genes is not included. In a few studies, where TSC1 and TSC2 promoter region analysis has been performed, the levels of mutations detected were either very low or null. The main reasons for exclusion of the above mentioned genomic areas in usual TSC genetic analysis are the limitations imposed by direct Sanger sequencing, but also the fact that in the majority of the patients, pathogenic mutations are detected in the exons and the intron/exon boundaries of the TSC genes.

Nevertheless, nowadays, there is a trend towards the use of NGS-based technologies in TSC genetic analysis, exactly due to the fact that these new methods have the ability to cover readings of the whole length of TSC1 and TSC2 genomic areas, including the promoters, UTRs, and whole intron sequences, but also because of their sensitivity, where mutations can be detected in the presence of even only 9% of the minor allele. Of course, on the other hand, the use of NGS-based genetic analysis cannot solve the problem of the variants of unknown clinical significance (VUS) and eliminate the need for functional analysis.

Although the major contributions on TSC mutation scanning have been based on Sanger sequencing and less than 10 NGS papers have appeared in the TSC literature until this day, one cannot ignore the advantages of NGS in TSC genetic analysis over Sanger sequencing, which is posing some inherent limitations in our study.
Conclusions

In this study, TSC1 and TSC2 disease percentages were rather similar (46% vs 54%), but detection of mutations proved more effective in patients having definite TSC (85%) than in patients having a possible TSC diagnosis (29%). Five new mutations were identified, while TSC1 disease presented with a milder phenotype, consistent with previous reports. Most TSC2 mutations identified were de novo (86%). This was probably due to the more severe TSC2 disease phenotype, which likely prevents these individuals from having a family. In agreement with the above, most familial cases (75%) had TSC1 disease, likely due to its milder phenotype. The same was observed for all the patients with possible TSC diagnosis (100%), and the single family with multiple affected members. Nevertheless, familial cases could be slightly underrepresented in our study, since in some families only one of the parents was available for genetic testing. Finally, because TSC is one of the few rare diseases for which a targeted drug therapy is available, a more accurate genetic testing protocol should be introduced in order to help uncover the underlying molecular events in NMI individuals. A closer collaboration of scientists with TSC patient groups worldwide will probably shed more light on genotype/phenotype correlations in the near future, in the direction of improving the quality of life of patients suffering from TSC and families.

Patients and Methods

Patients. The study protocol and the informed consent forms were approved by the Bioethics Committee of NCSR "Demokritos". All patients referred to the laboratory of Environmental Mutagenesis and Carcinogenesis, Molecular Diagnosis of Genetic Diseases Project, for genetic testing. In four cases (families 7, 8, 9 and 11) prenatal diagnosis has also been performed. Before molecular diagnosis, written informed consent forms were signed by all probands or parents, whereas after analysis, families were informed in detail on the outcome of the genetic test. Finally, the study was in agreement with the 1975 Helsinki declaration, revised in 1983.

Mutation detection. Genomic DNA was extracted from peripheral blood lymphocytes according to the standard saturated salt-chloroform extraction protocol. Purity and concentration of isolated DNA were measured using a NanoDrop™ spectrophotometer, while the quality of genomic DNA was evaluated through agarose gel electrophoresis. The entire translated regions of TSC1 (exons 3 to 23) and TSC2 (exons 1 to 41) were PCR-amplified and then directly sequenced using the Sanger method. All primer sequences and PCR conditions used are shown in Tables S1 and S2 (see Supplemental Materials and Methods). Cycle sequencing reactions were performed using the v3.1 BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA), and then analysed on an ABI Prism™ Genetic Analyzer. Sequences obtained were aligned against reference sequences from the Genbank (Accession Numbers: NG_012386.1 (TSC1) and NG_005893.1 (TSC2)), and examined for the presence of variants. Family members of mutation carriers were being informed in counselling sessions, and if they consented, they were subjected to genetic analysis for the specific mutation. The origin of mutations (inherited or de novo) was inferred after testing both parents (when available).

Confirmation of the presence of an aberrant mRNA splice variant by RT-PCR analysis followed by DNA sequencing. In order to test for possible splicing anomalies within TSC2 mRNA transcripts (family 11), we performed total RNA extraction from peripheral blood lymphocytes, using TRI REAGENT (Molecular Research Center Inc, Cincinnati, OH). Subsequent cDNA synthesis was carried out using M-MLV RT (Life Technologies, Carlsbad, CA). In the particular family, RT-PCR analysis was performed with the help of a forward primer on exon 38 and a reverse primer on exon 41. RT-PCR products were sequenced and analysed on an ABI Prism™ Genetic Analyzer.

Multiplex ligation-dependent probe amplification (MLPA). Possible large rearrangements in TSC1 and TSC2 were assessed by Multiplex Ligation-dependent Probe Amplification (MLPA) using the SALSA MLPA P124 and SALSA MLPA P046, respectively, following manufacturer’s instructions (MRC-Holland, Netherlands).

Molecular Modeling of missense mutations. The 3D-model of the core domain of human TSC1 (aa: 1–262) was obtained from the Swiss_Model database and was based on the known crystal structure of the TSC1 core domain from S. pombe (PDB entry: 1KKO). The initial models of both the wild-type (wt) hTSC1 core domain and of its R1246K variant were subsequently subjected to molecular dynamics (MD) simulations in explicit water, using a procedure similar to that applied in Voukakis et al. 2016. Three independent, 50 ns long MD simulations were performed for each molecule. The 3D-model of the GAP domain of human TSC2 (aa: 1502–1756) was constructed using a combination of the Swiss-Pdb Viewer program and the Phyre server. The known crystal structure of the Rap1GAP catalytic domain was used as template, for this purpose.

Data availability. Data are available upon request.

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Author Contributions
S.A. was in contact with the TSC families, prepared Tables 1 and 2, prepared Figs 1 and 2, and carried out a significant part of the sequencing work. F.F. carried out part of the sequencing work, and part of the MLPA experiments. A.V. carried out part of the MLPA experiments. Y.N. carried out database searches and prepared Table 3. A.D. carried out obvious database searches and prepared Table 4. R.Vo. carried out database searches. R.Vr. carried out database searches. S.Y. provided part of the clinical data included in Table 1. D.J.S. was in contact with the TSC families and contributed to the writing of the manuscript. M.V. carried out prediction analysis for TSC1 R246K and TSC2 I1648F variants and prepared Figs 3 and 4. A.A. wrote part of the manuscript. D.Y. was in contact with the TSC families and the Tuberous Sclerosis Association of Greece, and supervised part of the work. G.E.V. was in contact with the TSC families and the Tuberous Sclerosis Association of Greece, designed the primers, supervised part of the work, coordinated the team and wrote the main manuscript text. All authors reviewed the manuscript.

Additional Information
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