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MOLECULAR-GENETIC CHARACTERISTICS AND GENOTYPE-PHENOTYPE CORRELATIONS IN BULGARIAN PATIENTS WITH TUBEROUS SCLEROSIS COMPLEX

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Abstract. Objective: *The aim of the study was to determine the molecular-genetic characteristics of the autosomal dominant systematic disorder Tuberous Sclerosis Complex (TSC1 and TSC2) in Bulgarian patients and to derive some genotype-phenotype correlations.* ***Material and Methods:*** *In total 42 patients/families with suspected clinical diagnosis of TSC were analyzed. We used direct sequencing and MLPA for the TSC1 and TSC2 gene analysis.* ***Results:*** *In 38 families (90.5%) we confirmed the suspected clinical diagnosis – 15 with TSC1 (35.7%) and 23 (54.8%) with TSC2. In 4 families (9.5%) pathogenic variants were not found. In all 38 patients with proven diagnosis of TSC, we found 38 different mutations, 15 of which (39%) were detected for the first time by our research group. The mutation "hotspots" in TSC1 gene are exons 9, 15, 17 and 18, where 73% of the TSC1 mutations are localized, while the TSC2 gene mutation "hotspots" are exons 13 and 34, with 22% of the mutations situated there. In the TSC2 patients the common clinical findings include subcortical tubers, epilepsy with generalized tonic-clonic seizures, subependymal giant cell astrocytoma, facial angiofibromas, ungual fibromas, cardiac rhabdomyomas and renal angiomyolipomas, while in the TSC1 patients typically cortical tubers, cortical dysplasia and subependymal nodules were registered. In patients with aggressive frameshift and nonsense TSC1 and TSC2 mutations commonly hypomelanotic macules, cortical and subcortical tubers, cortical dysplasia, epilepsy with different types of seizures were found. Renal angiomyolipomas and cysts were detected mainly in patients with large deletions.*

Shagreen patches and intellectual disability were typically registered in equal degree in patients with frameshift, nonsense and missense mutations. ***Conclusion:*** *Although some genotype-phenotype correlations were derived, there is a great inter- and intrafamilial clinical variability in TSC, so it is impossible to predict the course of the disease on the basis of the detected molecular defect. The obtained results helped us to develop a diagnostic algorithm for proper molecular-genetic diagnostics which permits adequate genetic counseling, prophylaxis and treatment in the affected TSC families.*

Key words: *tuberous sclerosis complex, TSC1 gene, TSC2 gene*

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INTRODUCTION

Tuberous sclerosis complex (TSC; TSC1, OMIM#191100 and TSC2, OMIM#613254) is an autosomal dominant disease with frequency about 1:6000 – 10 000 [3, 11]. TSC is characterized by hamartomatous tumors in multiple organs (brain, eyes, heart, kidney, skin and lungs). The leading causes of morbidity and mortality are tumors affecting the central nervous system and the kidneys [2, 3, 13].

TSC is due to mutations in one of the tumor suppressor genes – TSC1 (MIM#605284), encoding the protein hamartin or TSC2 (MIM #191092), encoding the protein tuberin, which results in hyperactivation of the mTOR signaling pathway, and subsequent cell proliferation.

In the present study, our resent results are combined with our previously published TSC1 and TSC2 results within the context of the patients' clinical data to evaluate the phenotype genotype correlation.

MATHERIALS AND METHODS

In total 42 patients/families with suspected clinical diagnosis of TSC were analyzed. Fifteen of the patients were reported in our previous study [6], while the rest 27 were tested in the present study. DNA samples were obtained from peripheral blood after written informed consent was obtained. The study was approved by the Ethics Committee of Sofia Medical University.

All patients were subjected to direct sequencing by BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) for mutation screening in both TSC1 and TSC2 genes. Patients with negative sequencing results were tested for changes in the copy number (large gene deletions/duplications) by Multiplex Ligation-Dependent Probe Amplification (MLPA) with SALSA MLPA P124 TSC1 probemix (MRC-Holland) for TSC1 gene and SALSA MLPA P046 TSC2 probemix (MRC-Holland) for TSC2 gene.

The electrophoretic separation of the MLPA and direct sequencing fragments was performed on ABI Prism 3130 Sequence Genetic Analyzer (Applied Biosystems) with polymer POP7 (Applied Biosystems). The electrophoretic data were automatically analyzed with ABI3130 Data Collection Software (Applied Biosystems, Foster City, CA) for the direct sequencing or GeneMapper Software v.4.0 (Applied Biosystems, Foster City, CA) for the fragment analysis.

The nucleotide changes in both genes were described according to the coding DNA reference sequence NM_000368.4 for human TSC1 and NM_000548.4 for human TSC2.

All identified genetic variants were described according to the Human genome Variation Society (HGVS) recommendations and were checked in two large mutation databases – Leiden Open Variant Database (http://www.lovd.nl/) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/).

The impact/pathogenicity of the unpublished gene variants were checked in VarSome (https://varsome.com/) where data from multiple predictors (MutationTester, Mutation assessor, FATHMM, FATHMM-MKL, FATHMM-XF, LRT, DEOGEN2, EIGEN, EIGEN-PC, SIFT, SIFT-4G, PROVEAN, MVP, REVEL, PrimateAL, MetaSVM, MetaLR, MutPred) is provided.

RESULTS

We confirmed the suspected clinical diagnosis of TSC in 38 (90.5%) of the 42 tested families – 15 with TSC1 (35.7%) and 23 (54.8%) with TSC2. In 4 families (9.5%) pathogenic variants were not found.

In the 15 TSC1 patients we found 15 different TSC1 mutations (4 frameshift and 11 nonsense) located in exons 5, 9, 15, 16, 17, 18, 21 and 22 (Fig. 1). Four (27%) of the detected mutations are described for the first time by our research group (2 mutations we report now, the other 2 we have already published [6]) (Table 1). About 27% of the detected TSC1 mutations are in the Rho-activating domain of hamartin, where the site for interaction with tuberin is situated; about 27% are in the coiled-coil domain, and about 20% are in the C-terminal end where hamartin interacts with the protein neurofilament light chain.

In the 23 TSC2 patients we found 23 different TSC2 mutations – 8 frameshift (two resulted from complex indel rearrangements), 6 missense and 4 nonsense (in exons 4, 11, 12, 13, 15, 19, 20, 26, 28, 34, 37, 38 and 39), 3 splice site mutations (in introns 9, 16 and 25), as well as 2 large deletions encompassing exons 1-16 and exons 15-42 respectively (Fig. 2). About half of the detected mutations (11 mutations, 48%) are described for the first time by our research group (8 mutations we report now, while the other 3 we have already published [6]) (Table 1). About 20% of the detected TSC2 mutations are in the hamartin binding domain of tuberin and about 35% are located in different functional domains for binding different proteins, like Pam (Protein associated with Myc), HPV16 E6 (Human papilloma virus E6 protein), CaM (Calmodulin), 14-3-3, rabaptin-5 (Rab GTPase binding effector protein 1), estrogen receptor alpha, 5'-AMP-activated protein kinase, Rheb, Rap1A, RalA, retinoic acid receptor alpha and vitamin D receptor.

The derived genotype-phenotype correlations in the present study were based on 33 Bulgarian genetically verified TSC cases (12 with TSC1 and 21 with TSC2) for whom detailed clinical description is available in our records (Table 2). In the TSC2 patients the common clinical findings include subcortical tubers, epilepsy with generalized tonic-clonic seizures (GTCS), facial angiofibromas and cardiac rhabdomyomas while in the TSC1 patients typically cortical tubers, cortical dysplasia and subependymal nodules were registered. Subependymal giant cell astrocytoma (SEGA), ungual fibroma and renal angiomyolipomas were detected only in TSC2 patients.

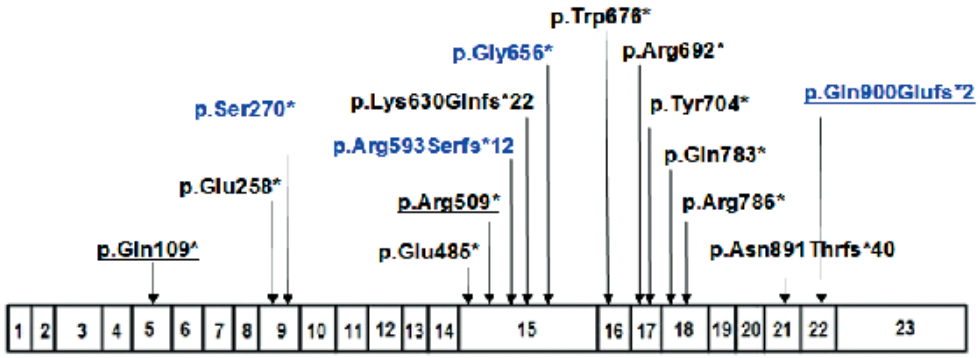

Fig. 1. TSC1 gene mutations in Bulgarian patients. With the numbered boxes the 23 TSC1 exons are schematically represented. The mutations marked in blue are described for the first time by our research group. The underlined mutations are already published by us [6]. The mutations known in the literature are given in black

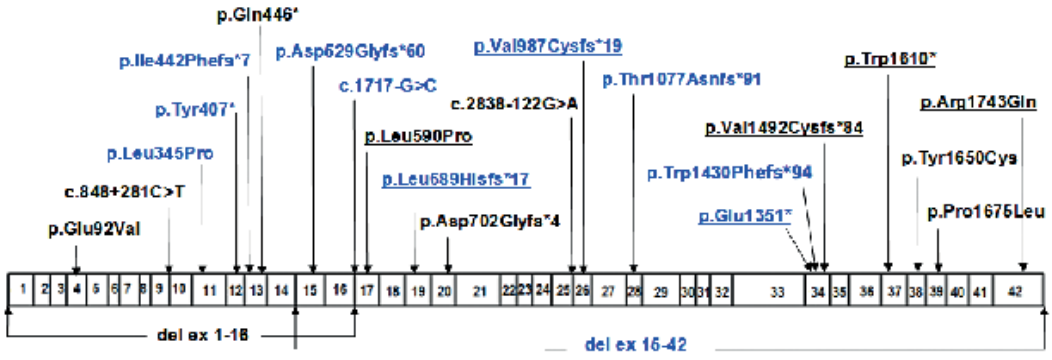

Fig. 2. TSC2 gene mutations in Bulgarian patients. With the numbered boxes the 42 TSC2 exons are schematically represented. The mutations marked in blue are described for the first time by our research group. The underlined mutations are already published by us [6]. The mutations known in the literature are given in black.

Table 1. TSC1 and TSC2 gene mutations in Bulgarian patients. The novel mutations are represented in blue

Mutation type	TSC1 mutations (n = 15)	TSC2 mutations (n = 23)
Frameshift	4 / 26.7% c.1779_1780delAG, p.Arg593Serfs*12 c.1888_1891delAAAG, p.Lys630Glnfs*22 c.2672delA, p.Asn891Thrfs*40 c.2698_2699delCA, p.Gln900Glufs*2	8 / 34.8% c.1324delA, p.Ile442Phefs*7 c.1583dupT, p.Asp529Glyfs*60 c.2066_2073del8,insACGGGCAGGGACCTCGCTGGGfs18, p.Leu689Hisfs*17 c.2105_2108delCTGA, p.Asp702Glyfs*4 c.2954_2957dupATGT, p.Val987Cysfs*19 c.3229dupA, p.Thr1077Asnfs*91 c.4289_4290delGGinsTTC, p.Trp1430Phefs*94 c.4473delA, p.Val1492Cysfs*84
Nonsense	11 / 73.3% c.325C>T, p.Gln109* c.325C>T, p.Gln109* c.809C>G, p.Ser270* c.1453G>T, p.Glu485* c.1525C>T, p.Arg509* c.1966G>T, p.Gly656* c.2027G>A, p.Trp676* c.2074C>T, p.Arg692* c.2111_2112delAT, p.Tyr704* c.2347C>T, p.Gln783* c.2356C>T, p.Arg786*	4 / 17.4% c.1221C>G, p.Tyr407* c.1336C>T, p.Gln446* c.4051G>T, p.Glu1351* c.4830G>A, p.Trp1610*
Missence	–	6 / 26.1% c.275A>T, p.Glu92Val c.1034T>C, p.Leu345Pro c.1769T>C, p.Leu590Pro c.4949A>G, p.Tyr1650Cys c.5024C>T, p.Pro1675Leu c.5228G>A, p.Arg1743Gln
Splice site	–	3 / 13% c.848+281C>T, p.Arg283Serfs*40 c.1717-1G>C c.2838-122G>A
Large deletions	–	2 / 8.7% c.(?_-106)_(1716+1_1717-1)del, deletion ex 1-16 c.(1443+1_1444-1)_(*102_?)del, deletion ex 15-42

Table 2. Clinical findings in 33 Bulgarian TSC patients – 12 with TSC1 and 21 with TSC2

Symptom	Total TSC1/2 (n = 33)	TSC1 (n = 12)	TSC2 (n = 21)
Hypomelanotic macules	28 (85%)	11 (92%)	17 (81%)
Facial angiofibromas	15 (45%)	4 (33%)	11 (52%)
Ungual fibroma	5 (15%)	–	5 (24%)
Shagreen patches	9 (27%)	3 (25%)	6 (29%)
SEGA	6 (18%)	–	6 (29%)
Cortical dysplasia/tubers	16 (48%)	8 (67%)	8 (8%)
Subependymal nodules	26 (79%)	11 (92%)	15 (71%)
Subcortical tubers	10 (30%)	2 (17%)	8 (38%)
Epilepsy	26 (79%)	9 (75%)	17 (81%)
GTCS	12 (36%)	3 (25%)	9 (43%)
Intellectual disability	16 (48%)	5 (42%)	11 (52%)
Cardiac rhabdomyomas	7 (21%)	1 (8%)	6 (29%)
Renal AML and cysts	6 (18%)	–	6 (29%)

Abbr.: SEGA – Subependymal giant cell astrocytoma; GTCS – Epilepsy with generalized tonic-clonic seizures; AML – angiomyolipomas

Some correlations may be found between the type of gene mutation (TSC1 or TSC2) and the clinical symptoms (Table 3).

In TSC1 patients the frameshift mutations were 100% associated with epilepsy and seizures of different types, which differed from nonsense mutations, causing seizures in 67% of the cases. At the same time, frameshift TSC1 mutations were not associated with subcortical tubers, severe epilepsy with GTCS, and intellectual deficits. Nonsense TSC1 gene mutations were more commonly associated with intellectual deficit (56%) and severe epilepsy (33%). In all TSC1 patients with cutaneous symptoms, the clinical findings were very similar and did not depend on the type of the mutation.

In TSC2 patients the splice site mutations were rarely found associated with hypomelanotic macules (33%) and were never found in combination with Shagreen patches, SEGA, cortical tubers, subcortical tubers, and mental retardation, compared to the rest of mutation types. Epilepsy and seizures of different types were 100% associated with frameshift mutations and large TSC2 gene deletions. SEN and hypomelanotic macules occured 100% in association with nonsense mutations and large gene deletions. Renal angiomyolipomas and renal cysts were 100% associated with large TSC2 gene deletions.

Furthermore, we present correlations between the clinical symptoms and the mutation type no matter if it is in TSC1 or TSC2 (Table 4). Our data revealed that hypomelanotic macules, cortical tubers, cortical dysplasia, subcortical tubers, epilepsy and different types of seizures, as well as severe epilepsy with GTCS, were more common in patients with frameshift and nonsense mutations, leading most probably to mRNA nonsense-mediated decay. Cardiac rhabdomyomas were more common in patients with frameshift mutations. In patients with large gene deletions, renal impairment was predominantly observed, while no ungual fibroma, Shagreen patches, and epilepsy with GTCS were ever detected. Shagreen patches and intellectual deficiency were observed at relatively similar rates in association to frameshift, nonsense and missense mutations. In patients with splice site mutations Shagreen patches, SEGA, intellectual deficiency, cortical dysplasia, cortical and subcortical tubers were not detected.

DISCUSSION

TSC2 accounts for about 55% of all TSC Bulgarian cases which sustains the published data, according to which 50-80% of all TSC cases are caused by mutations in the TSC2 gene [5, 7]. TSC1 accounts for about 36% of all TSC Bulgarian cases which is twice as much as the published data- 18% [5]. The proportion of our TSC cases with no mutation (9.5%) is in accordance with the literature – 5-15% [5, 7, 10].

Table 3. Distribution of the clinical findings in 33 Bulgarian TSC patients (12 with TSC1 and 21 with TSC2) depending on the type of the TSC1 and TSC2 gene mutations

	TSC1 (n = 12)		TSC2 (n = 21)				
Mutation / Symptom	Fsh (n = 3)	Nons (n = 9)	Fsh (n = 8)	Nons (n = 3)	Miss (n = 5)	Splice (n = 3)	Del (n = 2)
HM	3 (100%)	8 (89%)	7 (88%)	3 (100%)	4 (80%)	1 (33%)	2 (100%)
FA	1 (33%)	3 (33%)	3 (38%)	2 (67%)	3 (60%)	2 (67%)	1 (50%)
UF	–	–	2 (25%)	1 (33%)	1 (20%)	1 (33%)	–
ShP	1 (33%)	2 (22%)	2 (25%)	1 (33%)	3 (60%)	–	–
SEGA	–	–	2 (25%)	1 (33%)	2 (40%)	–	1 (50%)
CT/CD	2 (67%)	6 (67%)	3 (38%)	1 (33%)	2 (40%)	–	2 (100%)
SEN	3 (100%)	8 (89%)	5 (63%)	3 (100%)	3 (60%)	2 (67%)	2 (100%)
ST	-	2 (22%)	3 (38%)	2 (67%)	2 (40%)	–	1 (50%)
Epi	3 (100%)	6 (67%)	8 (100%)	2 (67%)	2 (40%)	1 (33%)	2 (100%)
GTCS	–	3 (33%)	5 (63%)	1 (33%)	2 (40%)	1 (33%)	–
ID	–	5 (56%)	5 (63%)	1 (33%)	4 (80%)	–	1 (50%)
CR	1 (33%)	–	2 (25%)	1 (33%)	1 (20%)	1 (33%)	1 (50%)
RI	–	–	1 (13%)	1 (33%)	1 (20%)	1 (33%)	2 (100%)

Fsh – frameshift mutation; Nons – nonsense mutation; Miss – missense mutation; Splice – splice site mutation; Del – big deletion; HM – Hypomelanotic macules; FA – Facial angiofibromas; UF – Ungual fibroma; ShP – Shagreen patches; SEGA – Subependymal giant cell astrocytoma; CT/CD – Cortical tubers, cortical dysplasia; SEN - Subpendimal nodules; ST – Subcortical tubers; Epi – Epilepsy of a different type; GTCS – Epilepsy with generalized tonic-clonic sizures; ID - Intellectual disability; CR – Cardiac rhabdomyomas; RI – Renal impairment (Renal angiomyolipomas and renal cysts)

Table 4. Correlation between the clinical symptoms and the mutation type in Bulgarian TSC patients

Mutation type / Symptom (n=number of patients)	Fsh	Nons	Miss	Splice	Del
Hypomelanotic macules (n = 28)	10 (36%)	11 (39%)	4 (14%)	1 (6%)	2 (7%)
Facial angiofibromas (n = 15)	4 (27%)	5 (33%)	3 (20%)	2 (13%)	1 (7%)
Ungual fibroma (n = 5)	2 (40%)	1 (20%)	1 (20%)	1 (20%)	–
Shagreen patches (n = 9)	3 (33%)	3 (33%)	3 (33%)	–	–
SEGA (n = 6)	2 (33%)	1 (17%)	2 (33%)	–	1 (17%)
Cortical dysplasia/tubers (n = 16)	5 (31%)	7 (44%)	2 (13%)	–	2 (13%)
Subependymal nodules (n = 26)	8 (31%)	11 (42%)	3 (11%)	2 (8%)	2 (8%)
Subcortical tubers (n = 10)	3 (30%)	4 (40%)	2 (20%)	–	1 (10%)
Epilepsy (n = 27)	11 (41%)	9 (33%)	4 (15%)	1 (4%)	2 (7%)
GTCS (n = 12)	5 (42%)	4 (33%)	2 (17%)	1 (8%)	–
Intellectual disability (n = 16)	5 (31%)	6 (36%)	4 (25%)	–	1 (6%)
Cardiac rhabdomyomas (n = 7)	3 (43%)	1 (14%)	1 (14 %)	1 (14%)	1 (14%)
Renal AML and cysts (n = 6)	1 (17%)	1 (17%)	1 (17%)	1 (17%)	2 (33%)

Abbr.: Fsh – frameshift mutation; Nons – nonsense mutation; Miss – missense mutation; Splice – splice site mutation; Del – big deetion; SEGA – Subependymal giant cell astrocytoma; GTCS – Epilepsy with generalized tonic-clonic sizures ; AML – angiomyolipomas.

In genetically not confirmed cases it could be suggested that the mutation is located outside the tested gene regions (for example deep in the introns) or somatic mosaicism, which could be proved by genetic testing on biopsy of the affected tissues.

We defined that the mutation “hotspots“ in the TSC1 is exon 15, where 33% of the mutations are situated, which is not surprising having in mind the large size of that exon, as well as exons 9, 17 and 18, where 40% of the mutations are detected (Fig. 1). In two large studies exon 15 [5, 8] and exon 18 [8] were also pointed as mutation “hotspots”.

The TSC2 mutation “hotspots“ are exons 13 and 34, where about 22% of the mutations are detected (Fig. 2). Exon 34 is pointed out for a mutation “hotspot“ by others as well [5].

The majority of TSC1 mutations detected in the Bulgarian sample were nonsense (73%) (Table 1), which is much higher than the reported data in the literature – 40-50% [5, 8]. Furthermore, the frameshift mutations account for 27%, which is in accordance with the literature- 20-30% [5, 8]. The absence of missense and splice site mutations, as well as large deletions or complex rearrangements sustains the numerous published data.

The majority of TSC2 mutations detected in the Bulgarian sample were frameshift (34.8%) (Table 1), which is close to some published data (38-40%) [8, 9], but higher than other data (23%) [5]. Furthermore, the missense mutations account for 26%, which is in accordance with the literature- 24-26% [5, 8, 9]. The frequency of our TSC2 nonsence mutations (17%) is close to some published data – 14.5% [9], but slightly lower than others – 21-25% [5, 8]. According to the literature [7] the most frequent TSC2 mutations have been frameshift and nonsense, which are equally distributed, while for the Bulgarian patients, as it became clear, the frameshift mutations were twice as common as the nonsence once. We detected splice site mutations (13%) more frequently than some authors – 8.5% [8], and less frequently than anothers – 16.6% [9]. We detected higher percentage of large gene deletions (8.7%) in comparison to the published data – 5-6% [5, 9].

The derived genotype-phenotype correlations based on the 33 Bulgarian TSC cases were compared with 5 large studies – 2 USA studies [1, 4], 2 China studies [5, 14] and 1 Dutch study [12].

In 79% of our patients epilepsy and seizures of different types were observed (see Table 2), which corresponds to the published data – 75-97%. The seizures were more frequently found in TSC2 (81%) compared to TSC1 patients (75%). Symptomatic epilepsy with generalized tonic clonic seizures (GTCS) were more common for TSC2 (43%) than for TSC1 (25%), which sustains the published data for more severe clinical presentation in TSC2 cases compared to TSC1 ones.

Cortical dysplasia and cortical tubers were found much more frequently in TSC1 (67%) than in TSC2 group (8%) or overall in the whole TSC patient group these findings were registered in 48% (see Table 2), which is a much lower percentage than the reported in the literature – 84-90%.

Subependymal nodules (SEN) were found in 79% of the patients (see Table 2), which correlated with the

published data (83-92%). Both cortical tubers and SEN were more commonly found in patients with TSC1 than in TSC2.

Subependymal giant cell astrocytoma (SEGA) was detected in 18% of all TSC patients (see Table 2), which correlates with the literature (11-30%), but in our group SEGA was presented only in TSC2 patients, which again supports the literature data for more severe clinical manifestation in TSC2 than in TSC1.

According to the literature, mental retardation varies widely (46-82%) and our data falls in this range 48%, but in TSC2 the percentage is slightly higher (52%) than in TSC1 patients (42%) (see Table 2).

Cardiac rhabdomyomas were registered much more frequently in TSC2 (29%) than in TSC1 patients (8%); the total percentage for TSC patients is 21% (see Table 2), which is lower compared to the literature (35%).

The cutaneous symptoms, like hypomelanotic macules were found at 85%, which is comparable with the literature (89-95%). In the TSC1 cohort, they are slightly more frequent (92%) than in TSC2 group (81%). Facial angiofibromas were found in 45%, which is in accordance with the literature (44-85%), being found to be more common in TSC2 patients (52%) than in TSC1 (33%). Shagreen patches were found in 27%, which is slightly lower than the published data (33-54%), and this symptom occurrs with equal frequencies in our TSC1 and TSC2 patients (see Table 2).

According to the literature, patients with TSC2 have more severe clinical symptoms than TSC1, but there are TSC1 patients with a severe phenotype as well. That conclusion is supported by our data that nearly 1/3 of the TSC1 Bulgarian patients have mental retardation, abnormalities in the central nervous system and seizures. On the other hand, we have two TSC2 cases with relatively mild clinical symptoms (patients do not have mental retardation and seizures), which sustain the published data on the broad clinical variability in TSC.

Despite the well-defined genotype-phenotype correlations in some aspects, there is considerable variability in the clinical symptoms between TSC patients. Genetic heterogeneity is also remarkable in our cohort: 38 different mutations in 38 genetically verified cases. It is even more difficult to establish a clear genotype-phenotype relationship, considering the presence of severe anticipation in some of the affected families. Our data reveals that patients with mild and severe phenotypes may have different as well as similar (or identical) genotypes, making it impossible to predict the course of the disease based on the detected molecular defect.

As a result of our work, we have introduced an optimal molecular genetic approach for the genetic diagnostics of patients with TSC (Fig. 3). The first step is sequencing of the mutation “hotspots” in both genes which confirms the diagnosis in 38% of all cases. Patients with negative results are subjected, to sequencing of the rest of the TSC2 exons and then the TSC1 exons, because mutations in the TSC2 gene occur at a higher frequency (54.8%) than those in the TSC1 gene (35.7%). In case we do not find mutations in either gene, we proceed with MLPA analysis to search for large deletions and duplications along both genes. As a final step, we perform sequencing for known pathological variants located deep within the introns of both genes.

CONCLUSION

Although some well-defined genotype-phenotype correlations in some aspects were derived, there is a considerable inter- and intrafamilial clinical variability in TSC, as well as high genetic heterogeneity, so it is

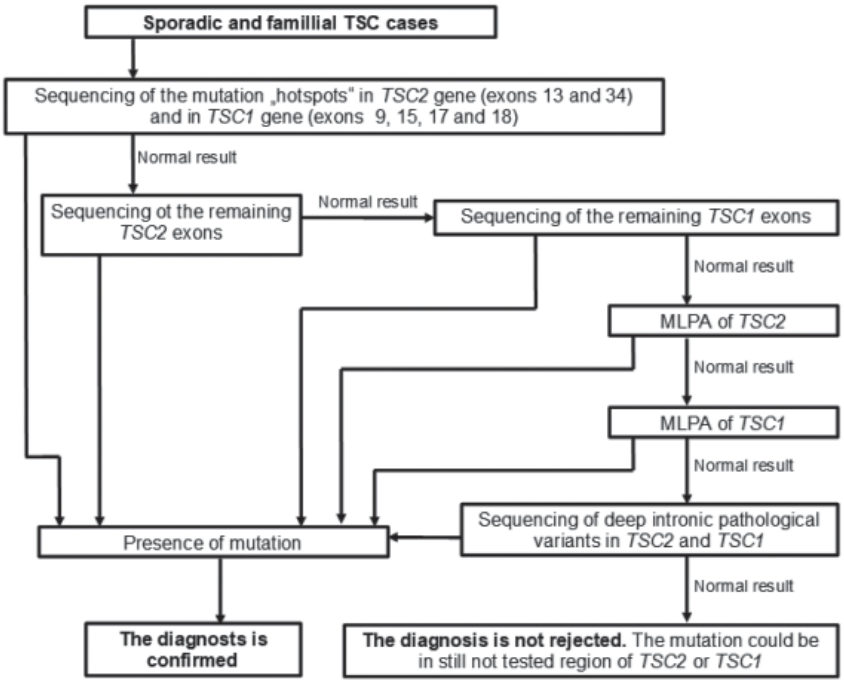

Fig. 3. Systematic algorithm for molecular-genetic analysis in patients with probable clinical diagnosis of TSC

impossible to predict the course of the disease on the basis of the detected molecular defect.

Our results helped us to propose a systematic diagnostic algorithm for fast and cost-effective genetic verification of TSC in Bulgarian patients. The proper molecular-genetic testing is a milestone in the management of TSC patients, ensuring that the affected families would receive adequate genetic counseling, prophylaxis and disease treatment.

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REFERENCES

1. Au KS, Williams AT, Roach ES, et al. Genotype/phenotype correlation in 325 individuals referred for a diagnosis of tuberous sclerosis complex in the United States. Genet Med. 2007;9,88-100.
2. Crino Pb, Nathanson Kl, Henske Ep. The Tuberous Sclerosis Complex. N Engl J Med. 2006; 355:1345-1356.
3. Curatolo P, Bombardieri R, Jozwiak S. Tuberous Sclerosis. Lancet. 2008;372:657-668.
4. Dabora SL, Jozwiak S, Franz DN, 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.
5. Ding Y, Wang J, Zhou S, et al. Genotype and Phenotype Analysis of Chinese Children With Tuberous Sclerosis Complex: A Pediatric Cohort Study. Front Genet. 2020;10;11:204.
6. Glushkova M, Bojinova V, Koleva M, et al. Molecular genetic diagnostics of tuberous sclerosis complex in Bulgaria: six novel mutations in the TSC1 and TSC2 genes. J Genet. 2018;97(2):419-427.
7. Hasbani DM, Crino PB. Tuberous sclerosis complex. Handb Clin Neurol. 2018;148:813-822.
8. Jiangyi W, Gang G, Guohai S, et al. Germline mutation of TSC1 or TSC2 gene in Chinese patients with bilateral renal angiomyolipomas and mutation spectrum of Chinese TSC patients. Aging (Albany NY). 2020;12(1):756-766.
9. Northrup H, Koenig MK, Pearson DA, et al. Tuberous Sclerosis Complex. In: Adam MP, Ardinger HH, Pagon RA et al, editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2020. 1999 Jul 13 [updated 2018 Jul 12].
10. Peron A, Au KS, Northrup H. Genetics, genomics, and genotype-phenotype correlations of TSC: Insights for clinical practice. Am J Med Genet C Semin Med Genet. 2018;178(3):281-290.
11. Roach Es, Gomez Mr, Northrup H. Tuberous Sclerosis Complex Consensus Conference: Revised Clinical Diagnostic Criteria. J Child Neurol. 1998;13(12):624-8.
12. Sancak O, Nellist M, Goedbloed M, et al. Mutational analysis of the TSC1 and TSC2 genes in a diagnostic setting: genotype–phenotype correlations and comparison of diagnostic DNA techniques in Tuberous Sclerosis Complex. Eur J Hum Genet. 2005;13, 731–741.
13. Weiner DM, Ewalt DE, Roach ES, et al. The tuberous sclerosis complex. A comprehensive review. J Am Coll Surg. 1998; 187:548-61.
14. Yang G, Shi ZN, Meng Y, et al. Phenotypic and genotypic characterization of Chinese children diagnosed with tuberous sclerosis complex.Clin.Genet. 2017;91,764-768.