Journal of Human Genetics (2018) 63:597–604
https://doi.org/10.1038/s10038-018-0416-0

ARTICLE

Pattern of *TSC1* and *TSC2* germline mutations in Russian patients with tuberous sclerosis

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Received: 31 August 2017 / Revised: 7 December 2017 / Accepted: 19 January 2018 / Published online: 23 February 2018
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Abstract

Tuberous sclerosis (TS) is a rare autosomal-dominant genetic disease. TS is manifested by the development of multiple hamartomas, which affect brain, kidneys, retina, skin and other organs. This study aimed to reveal specific features of molecular epidemiology of TS in Russia. Blood DNA samples from 61 patients with definite ($n = 53$) or probable ($n = 8$) clinical diagnosis of TS were tested for mutations in *TSC1* and *TSC2* genes using Sanger sequencing and MLPA analysis. Five *TSC1/2* mutation-negative patients were further analyzed by exome sequencing. *TSC1/2* mutations were detected in 53/61 patients (87%): 39 (74%) carried mutations in the *TSC2* and 14 (26%) in the *TSC1*. Large rearrangements (exon deletions/duplications) affected exclusively *TSC2*, accounting for 15% of lesions of this gene. 6/8 (75%) patients with incomplete clinical manifestation of TS carried *TSC1/2* gene lesion. Overall, 96% of detected germline *TSC1/2* mutations occurred de novo. Patients with no mutation identified (NMI) differed from *TSC1/2* mutation carriers, being lacking cortical tubers and subependymal nodules but having higher frequencies of renal angiomyolipomas, rhabdomyomas, and lymphangioleiomyomatosis. Exome sequencing failed to identify overt disease-causing mutation candidates among NMI patients. Russian patients with TS have increased frequency of *TSC2* large gene rearrangements and *TSC1/2* mutations occurring de novo as compared to other studies. Patients with suspected TS diagnosis but NMI status may represent a distinct disease entity.

Introduction

Tuberous sclerosis (TS) is a rare autosomal-dominant genetic disorder affecting the skin, brain, heart, kidneys, lungs, eyes, and other organs [1]. The first TS locus was mapped to the distal long arm of chromosome 9 due to co-inheritance of this disease with ABO blood groups in some of the analyzed families [2]. This led to subsequent identification of the corresponding gene, *TSC1*, located on 9q34 and coding for hamartin [3]. The success in the discovery of the second TS locus was attributed to the emphasis on the specific feature of TS, i.e., presence of kidney cysts. While studying *TSC1*-negative TS pedigrees, Kandt et al. [4]. intentionally focused on the polymorphic markers located in the vicinity to the polycystic kidney disease gene (*PKD1*), and mapped the *TSC2* to chromosome 16p13.3. *TSC2* gene is considerably larger than *TSC1* and codes for tuberin [5, 6].

Both *TSC1* and *TSC2* act as negative regulators of mTOR signaling pathway. Their inactivation results in the appearance of multiple tumor-like lesions, hamartomas. In addition to acting as tumor suppressors, *TSC1* and *TSC2* are likely to play a role in a number of other biological

Electronic supplementary material The online version of this article (https://doi.org/10.1038/s10038-018-0416-0) contains supplementary material, which is available to authorized users.

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Table 1 *TSC1/2* mutation types [11–16, 21, 29–41]

Mutation type	*TSC1*		*TSC2*	
	This study	Other reports	This study	Other reports
Gain of stop codon	8 (57%)	18–45%	6 (15%)	5–36%
Frameshift	6 (43%)	43–55%	8 (20%)	12–45%
In-frame deletion	0	0–1%	1 (3%)	0–20%
Splice site alteration	0	7–18%	9 (23%)	7–20%
Missense mutation	0	0–9%	7 (18%)	17–20%
Large deletion/ duplication	0	0–0.5% in most populations studied [12–14, 16, 17, 21, 37, 40]; 3.2% in Japanese [41], and 9% in Korean [32] studies	6 (15%)	1–11% [12–14, 16, 17, 21, 32, 37, 40, 41]
	14 (26%)	7–56%	39 (74%)	44–93%

processes, e.g., in the functioning of nervous system. TS manifestations are highly variable, being dependent on the patient age and gender, type of the *TSC1/2* gene mutation as well as other, yet unrecognized factors. Most of currently diagnosed TS patients have a relatively severe disease presentation, which significantly alters their well-being. For this reason, subjects with TS have reduced chances to have a family, and therefore only less than a third of TS cases are caused by vertical transmission of the *TSC1/2* germline mutation [7]. The majority of TS instances are sporadic and attributed to de novo defect in the *TSC1* or *TSC2* genes. In addition, *TSC1/2* mutation mosaicism contributes to a share of TS morbidity [1, 7–9].

Clinical diagnosis of TS is based on the evaluation of so-called "major" and "minor" TS features. Major features include characteristic abnormalities of the skin and oral cavity (hypomelanotic macules, angiofibromas, ungual fibromas, shagreen patch), brain (cortical dysplasias, subependymal nodules, subependymal giant cell astrocytoma), heart (cardiac rhabdomyoma), eyes (multiple retinal hamartomas), lungs (lymphangioleiomyomatosis), and kidneys (angiomyolipomas). Confetti skin lesions, dental enamel pits, intraoral fibromas, multiple renal cysts, retinal achromic patches, and non-renal hamartomas are classified as minor TS features. Clinical TS diagnosis is considered definite if a patient has two major features of TS or combination of 1 major feature with at least two minor features. Subjects with only one major TS feature or two and more minor features belong to the category of possible TS diagnosis. Patients with genetic diagnosis of TS, i.e., presence of clearly pathogenic mutation in *TSC1* or *TSC2* gene, are classified as TS cases irrespectively of the clinical disease manifestation [10].

TS molecular genetics is relatively well described, with hundreds of patients already subjected to comprehensive *TSC1/2* gene testing. However, the geography of TS genetic studies is limited to a number of countries, including USA, Western Europe (UK, Netherlands, Germany), Poland, India, Taiwan, Korea, China, Japan and Australia (Supplementary Table 1). The existence of ethnic and/or country-specific variations in TS presentation is acknowledged in some studies, therefore the analysis of yet unstudied populations is justified [11]. Here we report the first Russian study describing the pattern of *TSC1/2* mutations in TS patients.

Materials and methods

Sixty one patients with clinical signs of tuberous sclerosis (TS) were collected during years 2012–2016 in several medical facilities operating in Moscow, St. Petersburg, Ekaterinburg and Ufa; the majority of patients were from the TS registry maintained by the Center of Epileptology (N.I. Pirogov National Research Medical University, Moscow, Russia). Fifty-three (87%) of recruited patients met criteria for definite diagnosis of TS, whereas the remaining 8 (13%) were classified as possible TS cases. The mean age of patients was 8.8 years (range: 3 months – 43 years). Informed consent was obtained for all the patients or their parents or guardians prior to their inclusion in the study. The study was approved by the local Ethics Committee.

Small mutations in *TSC1* and *TSC2* genes were analyzed by high-resolution melting (HRM) analysis followed by Sanger sequencing of suspicious fragments. Primer sequences and PCR conditions are presented in the Supplementary Table 2. Detection of large rearrangements was carried out by multiplex ligation-dependent probe amplification (MLPA) using P124 probemix for *TSC1* and P046 for *TSC2*; suspected *TSC2* alterations were confirmed using additional P337 probemix. The exact description of the MLPA protocol is available from the web site of the kit manufacturer (MRC Holland, http://www.mlpa.com).

DNA samples from five *TSC1/2* mutation-negative patients were subjected to whole-exome sequencing (WES). Exome enrichment was performed using the

Table 2 Comparison of clinical characteristics in *TSC1/2* mutation carriers and NMI patients

Characteristics	*TSC1* ($n = 14$)	*TSC2* ($n = 39$)	NMI ($n = 8$)	Total ($n = 61$)	Correlations (Fischer exact test)	Adjusted p value (Benjamini–Hochberg procedure)
Age						
Mean age at diagnosis, years (range)	9.8 (3 months – 43 years)	5.5 (3 months – 28 years)	23.2 (9 months – 37 years)	8.8 (3 months – 43 years)		
Median age, years	7.5	3.0	30.0		*TSC1* vs. *TSC2*: $p = 0.004$	*TSC1* vs. *TSC2*: $p = 0.006$
					TSC1 vs. NMI: $p = 0.035$	*TSC1* vs. NMI: $p = 0.035$
					TSC2 vs. NMI: $p = 0.002$	*TSC2* vs. NMI: $p = 0.006$
Gender						
Female	11 (79%)	18 (46%)	5 (63%)	34 (56%)	ns	
Male	3 (21%)	21 (54%)	3 (37%)	27 (44%)		
Neurology						
Seizures	11 (73%)	26 (67%)	2 (25%)	39 (63%)	*TSC1* vs. NMI: $p = 0.0260$	ns
					TSC2 vs. NMI: $p = 0.0472$	
Autism/autism-like features	1 (7%)	5 (13%)	1 (13%)	7 (11%)	ns	
Speech delay	1 (7%)	12 (31%)	0	13 (21%)	ns	
Brain						
Subependymal nodules	2 (13%)	9 (23%)	0	11 (18%)	ns	
Cortical tubers	7 (47%)	10 (26%)	0	17 (27%)	*TSC1* vs. NMI: $p = 0.0225$	ns
Subependymal giant cell astrocytoma (SEGA)	2 (13%)	6 (15%)	0	8 (13%)	ns	
Kidney						
Multiple renal cysts	2 (13%)	9 (23%)	0	11 (18%)	ns	
Renal angiomyolipomas	2 (13%)	12 (31%)	5 (63%)	19 (31%)	*TSC1* vs. NMI: $p = 0.0524$	ns
Skin						
Multiple hypomelanotic macules (3 or more lesions with diameter exceeding 5 mm)	11 (79%)	27 (69%)	2 (25%)	40 (66%)	*TSC1* vs. NMI: $p = 0.0260$	ns
					TSC2 vs. NMI: $p = 0.0407$	
Scanty hypopigmented macules (<3 lesions)	1 (7%)	2 (5%)	1 (13%)	4 (6%)	ns	
Facial angiofibromas	2 (13%)	12 (31%)	4 (50%)	18 (30%)	ns	
"Confetti" skin lesions	1 (7%)	0	0	1 (2%)	ns	

Table 2 (continued)

Characteristics	*TSC1* ($n = 14$)	*TSC2* ($n = 39$)	NMI ($n = 8$)	Total ($n = 61$)	Correlations (Fischer exact test)	Adjusted p value (Benjamini–Hochberg procedure)
Forhead fibrous patch	1 (7%)	3 (8%)	2 (25%)	6 (10%)	ns	
Shagreen patch	2 (13%)	5 (13%)	0	7 (11%)	ns	
Ungual fibroma	2 (13%)	2 (5%)	1 (13%)	5 (8%)	ns	
Hair						
White forelock	0	2 (5%)	2 (25%)	4 (6%)	ns	
Liver						
Liver angiomyolipomas	0	4 (10%)	1 (13%)	5 (8%)	ns	
Heart						
Cardiac rhabdomyoma	4 (27%)	23 (59%)	1 (13%)	28 (45%)	*TSC2* vs. NMI: $p = 0.0226$	ns
Lung						
Lymphangioleiomyomatosis (LAM)	0	2 (5%)	3 (38%)	5 (8%)	*TSC1* vs. NMI: $p = 0.0364$ *TSC2* vs. NMI: $p = 0.0289$	ns
Eye						
Retinal hamartomas	0	7 (18%)	1 (13%)	7 (11%)	ns	

NMI no mutation identified, *ns* nonsignificant ($p > 0.05$)

Nextera kit (Illumina, USA), which covers 37 Mb of coding sequences (214,405 exons; 98.3% of sequences annotated in RefSeq database). Massive parallel sequencing was carried out using an Illumina MiSeq instrument and included multiple 150-bp reads with approximately 50× coverage. The analysis of nucleotide-specific fluorescent signals was done using MiSeq Reporter software. Reads were aligned to the Human Reference Genome (version hg19) by Burrows-Wheeller Aligner. The obtained files were analyzed using GATK (Genome Analysis Tool Kit) software. The identified differences from the reference sequence were annotated using Annovar resource (www.openbioinformatics.org/annovar/).

Results

Mutational findings

In total, *TSC1/2* mutations were identified in 53/61 (87%) cases, which corresponds well to the majority of similar investigations [12–15] (Supplementary Table 1). The distribution of *TSC1/2* alterations is presented in Table 1. As in other reports, *TSC2* mutations were significantly more common than *TSC1* gene lesions. It is essential to acknowledge a significant contribution of large rearrangements of *TSC2* gene, which accounted for 15% of *TSC2* gene lesions, 11% of the total number of *TSC1/2* mutations and 10% of studied TS patients; these frequencies are markedly higher than in published studies [16, 17]. Combination of Sanger sequencing and MLPA allowed to reveal pathogenic mutations in 52 out of 61 patients. In addition, one previously missed *TSC2* mutation was identified among five samples analyzed by WES. Re-evaluation of the original HRM protocol revealed that this was entirely a human error. Examples of WES-driven detection of *TSC1/2* gene lesions overlooked by conventional genetic testing have already been described in the literature [18].

Individual clinical and genetic data for TS patients are presented in Supplementary Table 3. Several patients had hot-spot mutations in *TSC2* gene. Two patients (7131, MG279) carried *TSC2* c.138+1G>A allele. Another two subjects (6910, MG472) had large deletions encompassing exon 37-42 of *TSC2* gene as well as a part of neighboring *PKD1* gene. In addition two cases of TS (7323, MG393) were associated with *TSC2* c.1832G>A (p.R611Q) mutation. All these mutations were repeatedly described in prior studies [12, 16, 19–21]. Noticeably, these 6 *TSC2* mutations occurred de novo indicating that they represent rather the hot-spots than recurrent alleles persistent in the population; these findings are in agreement with some other reports [12], although familial cases for some of these mutations have been reported as well [13, 19, 21].

Genotype–phenotype associations

TSC1/2 gene mutations were detected in 47/53 (89%) and 6/8 (75%) patients with definite and possible diagnosis of TS, respectively. Comparison of clinical features of TS patients carrying *TSC1* mutation, *TSC2* gene lesion or no germline defects in these genes revealed some interesting trends (Table 2). Cases with no mutation identified (NMI) were older at diagnosis, while *TSC2*-associated patients had the youngest age at onset (*TSC1* vs. *TSC2*: $p = 0.004$; *TSC1* vs. NMI: $p = 0.035$; *TSC2* vs. NMI: $p = 0.002$); these data are in good agreement with other reports [12, 22, 23]. NMI patients lacked cortical tubers and subependymal nodules thus confirming the observations of Camposano et al. [24] and Boronat et al. [25]. In addition, NMI cases were characterized by statistically lower frequency of seizures, cardiac rhabdomyomas and multiple hypomelanotic macules. These observations fit well to the results of several prior studies, which acknowledged milder phenotype of NMI patients as compared to genetically proven TS cases [12, 14, 15, 21, 24]. On the other hand, NMI patients had significantly higher frequencies of renal angiomyolipomas and lymphangioleiomyomatosis; similar trends were reported by Staley et al. [22] and Camposano et al. [24].

TSC1 mutation carriers tended to have lower male-to-female ratio and lower frequency of present or past cardiac rhabdomyomas than patients with *TSC2* mutation, however these dissimilarities did not reach the threshold for statistical significance. Furthermore, only differences in the patients' age remained significant after the adjustment for multiple comparisons; however, good agreement with published studies indicate that the observed genotype–phenotype correlations are indeed characteristic for TS disease (Supplementary Table 4).

DNA samples from the parents were available for 46 patients. Overall, 44/46 (96%) tested negative for *TSC1/2* mutations identified in their children. Vertical transmission of potentially relevant mutation was documented only in 2/46 (4%) analyzed families. The TS-affected parent was detected only in one of these families (case 4972, Supplementary Table 2). In another family (MG306, Supplementary Table 2), the disease was apparently caused by *TSC2* c.1865G>C (p.R622P) mutation. This mutated allele was maternally transmitted, however the mother pretended to be healthy. There are some data suggesting that c.1865G>C (p.R622P) allele [26] and some other *TSC1/2* mutations [27] are associated with milder disease course. In another family (MG317), there were some clinical features of TS in the mother of the patient (facial angiofibromas and single hypomelanotic macule), however she tested negative for *TSC2* exon 26-27 deletions. This can be attributed to mosaic character of *TSC2* gene defect in this woman; mosaic

mutations are known to be poorly detectable by conventional MLPA assay [28].

Whole-exome sequencing

Five patients were subjected to exome sequencing analysis. Two of these patients had definite diagnosis of TS (5170 и MG187, Supplementary Table 2), whereas the remaining three were adult women with renal angiomyolipomas and lymphangioleiomyomatosis (6570, MG86, MG102, Supplementary Table 2). As stated above, exome sequencing revealed previously overlooked *TSC2* c.5227C>T (p. Arg1743Trp) in the patient MG187; this mutation is classified by LOVD database as probably pathogenic. We further analyzed genes, which are involved in the interaction with *TSC1*, *TSC2*, or *MTOR* according to BioGrid database. Twelve rare variants were identified; all were missense mutations and none occurred in more than one patient (Supplementary Table 5). MG102 carried potentially relevant *CCND2* c.455C>A (p.Ala152Glu) mutation; however, this mutation was also detected in healthy mother and sister of the proband, which argued against its pathogenicity. The above variants were not functionally assessed in the present work and require further investigation to clarify their implication in TS.

Discussion

This is the first study describing Russian patients with tuberous sclerosis. The pattern of *TSC1* and *TSC2* mutations was generally similar to other patient series [12, 14, 21, 29]. The direct comparison of overall frequencies of *TSC1/TSC2* gene defects between studies is complicated, given that the available reports utilized different techniques of mutation screening, distinct stringency of criteria for patient selection, and the clinical definitions of TS slightly evolved over the time [10, 21]. For example, Yamashita et al. [30] succeeded to detect *TSC1/TSC2* only in 37% of patients analyzed; however, the median age of the patients was unusually high (19.4 years), that favored bias towards NMI cases. On another end of interstudy variations, two groups reported the detection rate of 100% [31, 32]. Although the Korean study involved only 11 patients and therefore lacked sufficient statistical power [32], the reason of the lack of NMI cases in a series of 117 Chinese patients remains obscure [31].

Our study focused mainly on patients with definite diagnosis of TS, however 8 subjects with “possible” TS were included as well; 6 (75%) of the latter were found to have *TSC1/2* mutations, strongly indicating that even patients with incomplete clinical manifestation of TS should not be denied genetic testing. One could foresee that in the near future subjects with even minor symptoms of TS will be subjected to comprehensive *TSC1/2* mutation testing, thanks to decreasing costs and improving accessibility of the DNA analysis. It is of interest, whether these initiatives will result in the detection of high number of virtually asymptomatic patients with genetically proven diagnosis of TS. Increased diagnostic activities towards TS patients with latent disease appearance and therefore almost normal social fitness are also likely to lead to revealing of additional instances of familial clustering of *TSC1/2* mutations.

Another factor contributing to the differences in the mutation frequencies is the extent of *TSC1/TSC2* gene analysis. Given that the detection of *TSC1/TSC2* large rearrangements requires distinct experimental protocol and reagents, several investigators omitted the use of MLPA or similar techniques while analyzing genetic causes of TS [29, 30, 33–36]. As expected, incomplete *TSC1/TSC2* gene testing resulted in somewhat lower mutation rate, with the maximal estimate approaching to 76% in the study by Hung et al. [36]. Our investigation detected large gene rearrangements in 6/53 (11%) patients with germline *TSC1/TSC2* mutations. This is on the upper limit of interstudy variations, as the contribution or gross *TSC1/TSC2* alterations in the total mutation spectrum ranged from 1–2% [13, 14] to 11% [37]. Overall, *TSC1/2* large gene rearrangements make a noticeable contribution in TS morbidity. Therefore, the genetic analysis of TS patients should not be limited to Sanger sequencing, and MPLA testing has to be regarded as an absolutely mandatory part of examination of patients with suspected TS diagnosis.

TSC2 mutations were significantly more common in our patients than *TSC1* gene lesions. Virtually all available studies on TS patients demonstrated higher frequency of *TSC2* mutations as compared to *TSC1* alterations, with the ratio approaching to 3–3.5:1 [12–16, 21, 29, 31, 38, 39]. Interestingly, Japanese TS patients appear to have an elevated share of *TSC1* mutations [30, 34]; this trend does not strictly apply to other Asians, as *TSC1/TSC2* mutation ratio in Chinese [31], Taiwanese [36] and Malaysian [37] patient series resembled the one in Whites, and the Korean studies produced contradictory results [32, 35]. Similarly to other reports [12–14, 21, 36], all *TSC1* mutations in our study are truncating. In contrast, a substantial share of pathogenic *TSC2* alleles is represented by amino acid substitutions (Table 1 and refs. [13, 14, 16, 19, 21]).

The proportion of familial cases in our study is clearly lower as compared to other reports (5% vs. 11–38% [12, 13, 16, 21, 30–34, 37, 38, 40, 41]). This can be explained by poorer social adaptation of TS patients, underdiagnosis of milder forms of TS, or low frequency of TS germline mutations in the population. Noticeable clinical differences between NMI and *TSC1/2* mutation-positive patients also deserve attention (Table 2); the existence of unique

characteristics of the former indicates that NMI cases cannot be explained merely by technical failure of Sanger sequencing or MLPA. It is appealing to speculate that patients with NMI represent a distinct disease, which shows phenotypic overlap with TS but has different causes. It is important to realize that the age of patients may compromise the comparison of the NMI and mutation-positive patients. For example, cardiac rhabdomyomas in TS patients tend to resolve with time [42], whereas renal angiomyolipomas and lung lymphangioleiomyomatosis often manifest later than other TS symptoms [10]. Our study is retrospective by design, therefore many of included patients could have cardiac rhabdomyomas in the past, but returned to the normal heart status by the time of TS diagnosis. Similarly, many of included patients lack kidney and lung manifestations at present, but are likely to develop them in the future. NMI patients are evidently older than the subjects with proven genetic diagnosis of TS; it is difficult to figure out, whether a unique spectrum of clinical TS presentations in NMI cases is entirely attributed to distinct biological causes of the disease, or, at least in part, simply reflects an older age at TS diagnosis. There is a popular viewpoint among TS researchers suggesting that NMI cases cannot be explained by mutation in a novel TSC gene; instead, mosaic mutations in *TSC1/2* genes are detected in a subset of NMI patients [8, 43]. This corresponds well to the results of our exome sequencing study, which did not succeed to find clearly relevant germline mutations in NMI patients. However, it is important to keep in mind that WES technology has limited ability to detect gene rearrangements and therefore may miss some protein-coding mutations.

In conclusion, our study demonstrated high rate of *TSC1/2* mutations among clinically diagnosed Russian TS patients. A significant share of these mutations was represented by large gene rearrangements. The proportion of de novo *TSC1/2* mutations in our patient series was strikingly higher than in published studies. TS cases with no mutation identified had milder phenotype than subjects with detected *TSC1/2* gene lesion. Exome sequencing of NMI patients failed to reveal a novel TS-causing gene. Taken together, these data suggest that some of the patients with clinical diagnosis of TS but absence of germline mutations in *TSC1* and *TSC2* genes may represent a distinct disease entity.

Acknowledgements This work has been supported by the Russian Foundation for Basic Research (Grant No. 15-04-06714). Exome sequencing has been supported by the Russian Scientific Fund (Grant No. 15-15-00079).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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