PD 115677

Possible genetic backgrounds of tuberous sclerosis complex investigated by new generation techniques: biochemistry of genotype-phenotype association

BACKGROUND

Tuberous sclerosis complex (TSC) is an autosomal dominant rare disorder, which can affect one or more organ systems leading to a highly variable phenotype (Crino, Nathanson, & Henske, 2006). It causes tumor or hamartoma formation in the heart, kidney, skin, lung and brain (Curatolo, Bombardieri, & Jozwiak, 2008).

According to present knowledge TSC is caused by a mutations in *TSC1* (9q34) (van Slegtenhorst et al., 1997) or *TSC2* (16p13) (European Chromosome 16 Tuberous Sclerosis, 1993) genes, encoding the proteins hamartin (TSC1) and tuberin (TSC2). In 2012, a third component of the complex was discovered, the TBC1 domain family member 7 (TBC1D7) (Dibble et al., 2012). TBC1D7 stabilizes TSC the hamartin tuber complex through hamartin (Gai et al., 2016)

During routine molecular genetic diagnostic testing for *TSC1/TSC2* mutations, 15% of the TSC patients have any disease-causing mutation in these genes. In the scientific literature these patients are called “no mutation identified” (NMI) TSC cases. They have milder phenotype compared to patients with *TSC2* mutation, but similar to those patients with *TSC1* mutation and also renal involvement is also common in NMI patients (Sancak et al., 2005). The problem with NMI patients is, that in their case there is no explanation in the hands of the genetic counselors regarding the possible genetic and molecular background, which means they cannot give any satisfactory explanations for their symptoms, disease progression and inheritance type.

Next generation sequencing (NGS) has become the core technology for gene discovery in rare disorders. It has also the ability to increase the number of mutations identified in TSC patients (Metzker, 2010; Nellist et al., 2015). Whole Exome Sequencing (WES) technology focuses on the complete coding region of the genome, and scans thousands of genes in the same time.

AIM OF THE PROJECT

In this project we focused on Hungarian TSC NMI patients, in which no *TSC1*/*TSC2* mutations were identified using Sanger sequencing and multiplex ligation dependent probe amplification (MLPA). We used WES to find the clinically relevant genes and variants responsible for the described phenotypes.

METHODS

Study group: TSC NMI patients

Since 2011, the Department of Medical Genetics in Pecs carries out the molecular genetic analysis of *TSC1* and *TSC2* genes in Hungary. This biobank consists a total of 204 DNA samples (101 probands and 103 family members). From the 73 DNA proband samples analyzed with Sanger sequencing and MLPA, 9 patients (5 females and 4 males) were NMI cases, the others were either *TSC1* or *TSC2* positive. The 9 NMI DNA samples were further analyzed by WES, to find the possible disease-causing mutation(s).

Whole exome sequencing (WES)

The DNA samples of the 9 patients were processed in Centogene's laboratory (Germany). The company is CAP and CLIA certified, adheres to the "ACMG Recommendations for Reporting of Incidental Findings" and not reports on findings which are not directly related to the cause of a disease and not listed in the ACMG guidelines.

RNA capture baits against approximately 60 Mb of the Human Exome (targeting >99% of regions in CCDS, RefSeq and Gencode databases) was used to enrich regions of interest from fragmented genomic DNA with Agilent's SureSelect Human All Exon V6 kit. The generated library was sequenced on an Illumina platform to obtain an average coverage depth of ~100x. Typically, ~97% of the targeted bases are covered >10x. An end to end in-house Centogene bioinformatics pipeline including base calling, alignment of reads to GRCh37/hg19 genome assembly, primary filtering out of low-quality reads and probable artefacts, and subsequent annotation of variants was applied. All disease-causing variants reported in HGMD®, in ClinVar or in CentoMD® as well as all variants with minor allele frequency (MAF) of less than 1% in genomAD database were considered. Evaluation focused on coding

exons along with flanking +/-20 intronic bases. All inheritance patterns were considered. All identified variants were evaluated with respect to their pathogenicity and causality, and these were categorized into classes 1 – 6. All variants related to the phenotype of the patient, except benign or likely benign variants, were reported. Variants of relevance identified by NGS were continuously and individually in-house (Centogene) validated for quality aspects (Bauer et al., 2019). All variants reported in the manuscript had quality parameters that were shown to be indicative of a true positive NGS finding; Sanger confirmation was therefore omitted.

RESULTS

Molecular and clinical findings

From the 9 analyzed NMI patients, 6 patients received positive gene finding after WES. Table 1. shows the detailed genotype-phenotype correlations of the 9 NMI cases. All new, formerly not published variants were deposited into ClinVar (www.ncbi.nlm.nih.gov/clinvar) under accession numbers: SCV000902239: NM_001256071.2 (RNF213): c.2875G>T; p.(Gly959*), SCV000899291: NM_000368.4 (TSC1): c.232G>T; p.(Glu78*), SCV000899290: NM_000548.3(TSC2): c.226-6T>G and SCV000902495 NM_001134771.1 (SLC12A5): c.1417G>A; p.(Val473Ile)

Patient 1 had epilepsy and the MRI showed cortical tubers. WES revealed heterozygous nonsense mutations c.232G>T; p.(Glu78*) in *TSC1*. This variant is classified as likely pathogenic (class 2) and confirmed the genetic diagnosis of tuberous sclerosis type 1.

The second subject (*Patient 2*), presented renal tumors, angiofibromas on the cheeks, and ungual fibromas on the toes. Similarly to *Patient 1*, heterozygous nonsense mutation c.1498C>T; p.(Arg500*) was found in *TSC1*, which is classified as pathogenic (class 1). Both variants confirmed the genetic diagnosis of tuberous sclerosis type 1. Both *TSC1* variants create premature stop codon. The c.232G>T; p.(Glu78*) variant is formerly unpublished based on the online available TSC mutation databases, while the variant c.1498C>T; p.(Arg500*) has been previously described as disease causing for TSC by van Slegtenhorst and coworkers (van Slegtenhorst et al., 1999).

Patient 3 had epilepsy, cardiac benign tumor and the MRI results suggested TSC (cortical tuber). A heterozygous intronic variant in *TSC2* with uncertain significance (c.226-6T>G) was identified. It was predicted to disrupt the highly conserved acceptor splice site of exon 4. It is classified as variant of uncertain significance (class 3) leading to the genetic

diagnosis of tuberous sclerosis type 2 possible. Potential effect on splicing is routinely analyzed at Centogene using several in silico prediction programs. For the c.226-6T>G variant in TSC2, these programs predicted a negative/detrimental effect on the splice acceptor. “Splice Site Finder” assigned a splice acceptor score of 74.93 to the wild-type sequence (max value =100.00), while not at all scoring the mutant sequence.

In the case of 3 other patients WES revealed disease causing mutations in *PKHD1*, *SLC12A5* and *RNF213.* Based on the clinical features of these patients, specific attention was paid during WES to the genes *TSC1* and *TSC2*, but no relevant variants were found. However, pathogenic variants cannot be completely excluded since not all exons were fully covered due to limitations of the method.

Patient 4’s clinical signs included polycystic kidney, epilepsy and the MRI showed numerous typical signs of TSC (cortical tubers). Two heterozygous missense variants: c.5513A>G; p.(Tyr1838Cys) and c.3747T>G; p.(Cys1249Trp) were found in *PKHD1*. Both variants are classified as pathogenic (class 1) confirming polycystic kidney disease type 4. Both variants found in this gene were previously described as disease causing mutations for polycystic kidney disease (Rossetti et al., 2003; Ward et al., 2002). Since the *PKHD1* mutations can only partially explain the phenotype of the patient, whole genome sequencing (WGS) is being considered to uncover genetic variants not covered by WES. The establishment of genotype–phenotype correlations for *PKHD1* is not simple (Bergmann et al., 2003), and there are some phenotypic overlaps between ARPKD other diseases, among this TSC, which suggests functional relationship between the causative genes/proteins and the signaling pathway, in this case the mTOR (Huber, Walz, & Kuehn, 2011). Polycystin-1 and the TSC1/TSC2 tumor suppressor complex both act to suppress the activity of mTOR resulting in apoptosis (Shillingford et al., 2006). In our case, the common symptom between ARPKD and TSC was the renal cyst, however *PKHD1* mutations can only partially explain the phenotype of the patient. Based on the WES results we do not have the genetic explanation neither to epilepsy nor cortical tubers. WGS is more powerful tool compared to detect known genetic variants, small insertions/deletions and CNVs within the regions of the genome covered by WES. Given the fact that PKHD1 mutations can only partially explain the phenotype in the patient, we think WGS might be considered to uncover genetic variants not covered by WES. WGS typically results in ~10-20% additional genetic diagnoses.

Patient 5 presented hypomelanotic macule, epileptic seizures and white matter lesions as clinical features. Specific attention was paid during WES to early infantile epileptic encephalopathy (EIEE) related genes (*AARS, ALG13, ARHGEF9, ARV1, ARX, CACNA1A,*

CDKL5, DNM1, DOCK7, EEF1A2, FRRS1L, GABRA1, GABRB3, GNAO1, GRIN2B, GUF1, HCN1, ITPA, KCNA2, KCNB1, KCNQ2, KCNT1, NECAP1, PCDH19, PIGA, PLCB1, PNKP, SCN1A, SCN2A, SCN8A, SCN9A, SLC12A5, SLC13A5, SLC1A2, SLC25A12, SLC25A22, SLC35A2, SPTAN1, ST3GAL3, STXBP1, SZT2, TBC1D24, WWOX, AP3B2, KCNT2, HNRNPU, CAD, UBA5, FGF12, GABRB1, MDH2, YWHAG, DENND5A, SCN1B, GRIN2D, SYNJ1, SIK1), to *TSC1* and *TSC2* and to *IKBKG*-related disorder. WES analysis could not detect any relevant variant in these genes except in *SLC12A5*, where a heterozygous missense mutation c.1417G>A; p.(Val473Ile) with uncertain significance (class 3) was found. The variant is linked to cause susceptibility to idiopathic generalized epilepsy type 14 (EIG14). However, other pathogenic variants cannot be completely excluded since not all exons were fully covered due to limitations of the method.

Patient 6 showed epilepsy (epileptic seizures but the exact starting point could not be determined) and small hypopigmented spots. The MRI results suggested the possibility of TSC (cortical lesions). A heterozygous nonsense variant c.2875G>T; p.(Gly959*) in *RNF213* was identified The variant is classified as likely pathogenic (class 2). Pathogenic variants in the *RNF213* have been associated with susceptibility to Moyamoya disease type 2, which is probably multifactorial and polygenic disease with various clinical presentations and inheritance type. However, pathogenic variants cannot be completely excluded since not all exons were fully covered due to limitations of the method. Similarly to *Patient 4*, WGS is being considered to uncover genetic variants not covered by WES to find genetic variants to explain the remaining symptoms.

In the remaining 3 patients WES could not reveal any variants clinically relevant to the described phenotypes of these patients**.** It is important to mention, that pathogenic variants at these three patients cannot be completely excluded, since not all exons were fully covered due to limitations of the method. In *Patient 7*, who had renal angiomyolipoma which led to right kidney nephrectomy; multiplex angiomyolipomatosis in the left kidney, adenoma sebaceum on the cheeks and fibromas on the toes. During the analysis of her sample, specific attention was paid during WES to the genes associated with multiple endocrine neoplasia (*CDKN1B, RET, MEN1*) and TSC genes, but no variants clinically relevant to the described phenotype of the patient were observed. The psychomotor development of *Patient 8* was slow, MRI showed reduction in the volume of paraventricular white matter and right-side dominant polymicrogyria at the area of aqueductus cerebri. Front part of both temporal lobes and the frontal lobes were affected by polymicrogyria with moderate severity. He also had vermis hypoplasia. As dermatological signs halo nevus, one leaf-shaped hypomelanotic macule on the torso, one palm

size at the right knee bend were observable. In Patient 8 based on the clinical information specific attention was paid to the genes in neuronal migration disorders panel (*ACTB, ACTG1, ARFGEF2, ARX, COL18A1, COL4A1, CPT2, DCX, EMX2, EOMES, FGFR3, FH, FKRP, FKTN, FLNA, ADGRG1, IER3IP1, ISPD, LAMA2, LAMC3, LARGE, MED12, MEF2C, OCLN, PAFAH1B1, PAX6, PEX7, POMGNT1, POMT1, POMT2, PQBP1, RAB18, RAB3GAP1, RAB3GAP2, RELN, SNAP29, SRPX2, TUBA1A, TUBA8, TUBB2B, TUBB3, VDAC1, WDR62*) and on lissencephaly and brain malformation panel (*ACTB, ACTG1, ADGRG1, ARX, CDK5, COL6A1, COL6A2, COL6A3, DCX, DYNC1H1, EOMES, FKRP, FKTN, ISPD, KATNB1, KIF2A, KIF5C, LAMA2, LAMB1, LARGE, LMNA, NDE1, PAFAH1B1, POMGNT1, POMT1, POMT2, RELN, SELENON, TUBA1A, TUBB, TUBB2A, TUBB2B, TUBB3, TUBG1, VLDLR, WDR62, YWHAE*), but no relevant variants in these genes was found. WGS is considered which can add an additional 15-18% clarification rate compared to WES.

The MRI of *Patient 9* showed cortical tuber, SENs detected at the right side of the frontal ventricle and a cyst was detected at the area of the corpus pineale. Special attention was paid during WES to the *TSC1*/*TSC2*, but no variants clinically relevant to the described phenotype of the patient was detected.

Possible explanations of the genetic status of NMI patients

1.) Mutation detection failure during routine diagnostic. That was the case in 3 of our NMI patients (*Patients 1-3*).

2.) Mosaicism can be also an explanation based on the literature (example: (Tyburczy et al., 2015), so we considered mosaicism as a possible explanation for the genetic status of our TSC NMI patients. However, the WES analysis by Centogene confirmed mosaicism in any of our subjects.

3.) Mutations in introns affecting splicing, not near exons, promoter and enhancer regions which are not covered during routine molecular diagnostic testing. In one of our cases (*Patient 3*), intronic mutation was found.

4.) Possibility of a currently unknown TSC gene. According to our knowledge, there is any publication which confirms the presence of a new component of TSC which responsible for the disease. The routine pipeline of Centogene uses >25% allele frequency to detect mosaic variants.

SUMMARY AND CONCLUSION

We have identified with the use of WES in 6 TSC NMI cases the genes and mutations responsible for symptoms of the patients: in 2 cases previously unidentified *TSC1* mutation and in 1 patient an intronic variant of *TSC2* and disease-causing mutations in *PKHD1*, *SLC12A5* and *RNF21* genes in 3 other patients. In the remaining 3 patients WES could not reveal any variants clinically relevant to the described phenotypes.

We concluded that patients without the appropriate diagnosis due to the lack of sensitivity of the currently used routine diagnostic methods, or patients with unclear phenotype can significantly profit form the wider application of NGS technologies to find the genes and variants responsible for their symptoms to improve their treatment options and thereby their life quality. In agreement with other researcher groups (Nellist et al., 2015; Tyburczy et al., 2015), we think that with the use of NGS technologies during routine genetic diagnostics the number of NMI patients will be reduced.

ALTERATIONS FROM THE WORKPLAN

Whole exom sequencing (WES) and bioinformatics screening of the raw data of the NMI patients were performed with the involvement of Centogene (Germany). However, the chosen bioinformatic screening was unfortunately not enough. Because of this, more detailed bioinformatics analysis was needed, than the first one. This new analysis was performed by Centogene in the first quarter of 2018. Due to this delay of data processing and the longer publication process (which may last up to 6-8 months) of the results, an application for one more year extension of the project was submitted.

We prepared a manuscript entitled “Whole exome sequencing in a series of patients with a clinical diagnosis of Tuberous Sclerosis not confirmed by targeted TSC1/TSC2 sequencing”, which was uploaded to an appropriate English language scientific journal. We answered all the questions of the first-round review, but unfortunately the corrected manuscript was rejected by the editor of the journal. As the 5 new questions raised by the reviewer were mainly methodological and could be answered, we applied for another year extension for the project to publish our results in another journal. We rewrote the manuscript with a new title recommended by the reviewer: "Whole exome sequencing in a series of patients with a clinical diagnosis of Tuberous Sclerosis not confirmed by targeted TSC1/TSC2 sequencing” and uploaded it to another English-language scientific journal with an impact factor (Molecular Genetics &

Genomic Medicine IF: 1.995). The stage of the manuscript is in “Pending decision” since mid-July. Despite several email inquiries to the editorial office about the decision, any change in the status of the manuscript was made until today.

Because tissue samples from the NMI patients are not available, the planed analysis of tissue samples and functional analysis of the mutations identified during WES could not be performed.

PRESENTATIONS OF THE RESULTS

Genetic features and clinical diagnosis of tuberous sclerosis complex. Rare Disease Days in Pecs, February 25-27, 2016, Pécs (Oral presentation)

Experience of the last 5 years in the genetic diagnosis of tuberous sclerosis complex. Méhes Károly Genetic Training Days, October 11-13., 2017, Pécs (Oral presentation)

Kovesdi E., Hadzsiev K., Komlosi K., Horvath E., Horvath A., Farkas M.K., Farkas V., Timar L., Csaba A.K., Sumegi K., Melegh B; *Novel TSC1/TSC2 pathogenic variants in Hungarian cohort with Tuberous Sclerosis Complex: clinical and molecular genetic aspects*. (Mendelian Phenotypes, Program #993F). Presented at the 67th Annual Meeting of The American Society of Human Genetics, October 17–21. 2017, (Orlando, Florida, USA), (Abstract, Oral poster presentation)

Publications

Kövesdi E, Bene J, Nagy N, Horváth Á, Melegh B, Hadzsiev K. *Importance of gross deletions in the diagnosis of tuberous sclerosis complex: the first Hungarian cases*. Orv Hetil. 2017 Jul;158(30):1188-1194. doi: 10.1556/650.2017.30789. PMID: 28737457 Hungarian. IF: 0.349

Till Á, Szalai R, Hegyi M, **Kövesdi E**, Büki G, Hadzsiev K, Melegh B. *A rare form of ion channel gene mutation identified as underlying cause of generalized epilepsy*. Orv Hetil. 2019 May;160(21):835-838. doi: 10.1556/650.2019.31404. PMID: 31104500 Hungarian. IF: 0.497
(See in printed version the attached documentations of actions taken regarding of missing NKFI ID replacement)

Manuscript under publication process

Ripszam R, Postyeni E, Horvath BE, Kelemen A Fabos B, Farkas V, Hadzsiev K, Sumegi K, Magyari L, Moreno PL, Bauer P, Melegh B, **Kovesdi E**. *Whole exome sequencing in a series of patients with a clinical diagnosis of Tuberous Sclerosis not confirmed by targeted TSC1/TSC2 sequencing*. Molecular Genetics & Genomic Medicine IF: 1.995

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	Patient No.	P1	P2	P3	P4		P5	P6	P7	P8	P9
	Gender (M/F)	M	F	M	F		M	F	F	M	M
	Age (Y)	25	36	8	13		13	8	52	11	4
	TSC diagnostic status	*Possible*	*Definite*	*Definite*	*Possible*		*Possible*	*Possible*	Definite	Possible	Definite
Genetics	Affected gene	*TSC1*	*TSC1*	*TSC2*	*PKHD1*		*SLC12A5*	*RNF213*	NA	NA	NA
	c.DNA change	c.232G>T	c.1498C>T	c.226-6T>G	c.3747T>G	c.5513A>G	c.1417G>A	c.2875G>T			
	Protein change	p.Glu78X	p.Arg500X	N/A	p.Cys1249Trp	p.Tyr1838Cys	p.Val473Ile	p.Gly959X			
	Variant type	Nonsense	Nonsense	Splicing	Missense	Missense	Missense	Nonsense			
	Zygosity	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous			
	Classification	Likely pathogenic (2)	Pathogenic (1)	VUS (3)	Pathogenic (1)	Pathogenic (1)	VUS (3)	Likely pathogenic (2)			
	Diagnosis after WES	TSC type 1	TSC type 1	TSC type 2	Polycystic kidney disease type 4 with or without hepatic disease		Idiopathic generalized epilepsy type 14	Moyamoya disease type 2			
	Inheritance	AD	AD	AD	AR		AD	AD			
Skin	Hypomelanotic macule	-	-	-	-		+	+	-	+	-
	Adenoma sebaceum	-	+	-	-		-	-	+	-	-
	Ungual fibroma	-	+	-	-		-	-	+	-	-
	Shagreen patch	-	-	-	-		-	-	-	-	-
Central nervous system	SEN	-	-	-	-		-	-	-	-	+
	SEGA	-	-	-	-		-	-	-	-	-
	Cortical tuber	+	-	+	+		-	-	-	-	+
	Epilepsy	+	-	+	+		+	+	-	-	-
	Other	-	-	-	-		White matter lesions	Cortical lesions	-	Slow psychomotor development, Vermis hypolasia, Polymicrogiria	Corpus pineale cyst
Kidney	Renal cyst	-	-	-	+		-	-	-	-	-
	Renal tumor	-	+	-	-		-	-	-	-	-
	Angiomyolipoma	-	-	-	-		-	-	+	-	-
Heart	Ventricular septal defect	-	-	-	-		-	+	-	-	-
	Rhabdomyoma	-	-	+	-		-	-	-	-	-
Other symptoms		-	-	-	-		-	-	Lymphangioleio myomatosis	Right hallux onychodystrophy	-

Table 1. Genotype-phenotype features of TSC NMI patients. Abbreviations: *TSC1*: tuberous sclerosis 1, *TSC2:* tuberous sclerosis 2, *PKHD1:* fibrocystin, *SLC12A5:* solute carrier family 12 member 5, *RNF213:* ring finger protein 213, AD: autosomal dominant, AR: autosomal recessive, WES: whole exome sequencing, SEN: subependymal nodule, SEGA: subependymal glial cell astrocytome.