

## TUBEROUS SCLEROSIS COMPLEX SUPPRESSION IN CEREBELLAR DEVELOPMENT AND MEDULLOBLASTOMA: SEPARATE REGULATION OF MTOR ACTIVITY AND P27<sup>KIP1</sup> LOCALIZATION

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### Abstract

During development, proliferation of cerebellar granule neuron precursors (CGNPs), candidate cells-of-origin for the pediatric brain tumor medulloblastoma, requires signaling by Sonic hedgehog (Shh) and insulin-like growth factor (IGF), whose pathways are also implicated in medulloblastoma. One of the consequences of IGF signaling is inactivation of the mTOR-suppressing Tuberous Sclerosis Complex (TSC), comprised of TSC1 and TSC2, leading to increased mRNA translation. We show that mice in which TSC function is impaired display increased mTOR pathway activation, enhanced CGNP proliferation, GSK-3 alpha/beta inactivation, and cytoplasmic localization of the cyclin-dependent kinase (cdk) inhibitor p27<sup>Kip1</sup>, which has been proposed to cause its inactivation or gain of oncogenic functions. We observed the same characteristics in wild-type primary cultures of CGNPs in which TSC1 and/or TSC2 were knocked down, and in mouse medulloblastomas induced by ectopic Shh pathway activation. Moreover, Shh-induced mouse medulloblastomas manifested Akt-mediated TSC2 inactivation, and the mutant TSC2 allele synergized with aberrant Shh signaling to increase medulloblastoma incidence in mice. Driving exogenous TSC2 expression in Shh-induced medulloblastoma cells corrected p27<sup>Kip1</sup> localization and reduced proliferation. GSK-3 alpha/beta inactivation in the tumors *in vivo* and in primary CGNP cultures was mTOR-dependent, whereas p27<sup>Kip1</sup> cytoplasmic localization was regulated upstream of mTOR, by TSC2. These results indicate that a balance between Shh mitogenic signaling and TSC function regulating new protein synthesis and cdk inhibition is essential for normal development and prevention of tumor formation or expansion.

# POLYCYSTIN-1 REGULATES MTORC1 IN AN ERKS-DEPENDENT, AKT-INDEPENDENT MANNER *IN VITRO* AND *IN VIVO*

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## Introduction

Polycystin-1 (PC-1) the product of the gene most commonly mutated in Autosomal Dominant Polycystic Kidney Disease (ADPKD) regulates both proliferation and apoptosis, consistent with misregulation of both biological properties observed in ADPKD cystic epithelia. Several studies have recently linked the function of PC-1 to that of Tuberin. Furthermore, studies performed in several different animal models as well as on patients-derived tissues have revealed that the mTOR cascade is upregulated in Polycystic Kidney Diseases, although the precise mechanism of this dysregulation was not investigated.

We have recently shown using a series of loss and gain of function *in vitro* models that PC-1 controls cell growth (size) in addition to and independently of its action on proliferation. We have shown that PC-1 achieves this effect by inhibiting the mTORC1 pathway and its downstream effectors S6K and 4EBP1 in a *Tsc2*-dependent manner. Furthermore, PC-1 is able to regulate *Tsc2* by modulating its ERKs-specific phosphorylation in S664 (Distefano et al, MCB, 2009). Notably, we also have found that PC-1 induces full activation of Akt, suggesting a role of PC-1 in regulating mTORC2 as well, but this does not result in *Tsc2* phosphorylation in Akt-specific sites, suggesting that there is a disconnect between the PI3k/Akt pathway and mTORC1 in response to PC-1.

## Methods

In order to further investigate the mechanism by which PC-1 regulates the mTORC1 and 2 *in vivo*, we have generated a *bona fide* ADPKD mouse model by crossing a conditional mouse model generated in our laboratory (Wodarczyk et al., *submitted*), in which the last two exons of *Pkd1* are flanked by two loxP sites, with a mouse harbouring a kidney-specific Cre recombinase (Ksp-Cre, kindly provided by Dr. P. Igarashi, Southwestern University, TX-USA). *Pkd1 flox/-:Ksp-Cre* mice undergo kidney specific inactivation of the floxed allele and develop massive renal cysts after birth, ultimately leading to death by the second week of life

## Results

Biochemical analysis of lysates from polycystic kidneys at P4 and P8 shows hyperphosphorylation of downstream effectors of mTORC1, such as p70S6K and S6Rp as compared to controls. Furthermore, MEK1/2 and ERKs, but not Akt, are strongly upregulated, further suggesting that the ERKs, but not Akt control mTORC1 upregulation in ADPKD. Notably, immunohistochemistry on P4 cystic kidneys shows that the majority of cysts stain positive for phospho S6Rp (>70%), but we also observe a high proportion of cysts that do not show hyperactivation of the mTORC1 cascade.

## Conclusions

We propose a model by which PC-1 is able to regulate the mTORC1 cascade through an atypical mechanism that might be protective of cyst transformation, but this alone cannot justify renal cyst formation and expansion.

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## **SINGLE-CELL GENETIC REMOVAL OF TSC1 IN CORTICAL RADIAL GLIA**

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In Tuberous Sclerosis Complex (TSC) patients, mutations in one of two tumor suppressor genes, *TSC1* or *TSC2*, result in the formation of lesions. The mechanisms leading to TSC lesions and associated seizure generation during perinatal life remain unclear. The recent finding that radial glia act as neural progenitors led us to hypothesize that deletion of TSC genes in perinatal neural progenitor cells (i.e. radial glia) contributes to the generation of TSC lesions, and we propose to identify the mechanisms underlying that process.

To test our hypothesis, we genetically removed *Tsc1* in transgenic mice carrying conditional *Tsc1* alleles (fl) from embryonic progenitor cells using *in vivo* electroporation of a Cre recombinase-containing plasmid. We found that removal of *Tsc1* in subsets of cortical radial glia at embryonic day 16 (E16) did not disrupt the proper migration of cortical pyramidal neurons to layer II/III. However, *Tsc1*-null neurons displayed increased phosphorylation of S6 as expected following loss of functional Tsc1-Tsc2 complex and increased soma size. Next considering that TSC patients have a systemic loss of one *Tsc1* allele, in future experiments we will use a double hit model in transgenic mice carrying fl and mutant (mut, non-functional) *Tsc1* alleles. These data suggest that formation of TSC-like lesions such as cortical tubers requires using mice with a systemic loss of *Tsc1* prior removing the second allele.

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## MODULATION OF HYPOXIA INDUCIBLE FACTOR-1-ALPHA

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Hypoxia-Inducible Factor-1-alpha (HIF1-alpha) is a transcription factor essential for the maintenance of oxygen homeostasis. Under hypoxia, the HIF1-alpha subunit becomes stable enough to bind to the constitutively expressed beta subunit. It then translocates into the nucleus where it binds to HIF response elements located upstream of promoters on various target genes, thus triggering their gene expression. HIF1-alpha is known to regulate numerous targets which can in turn modulate cellular processes to increase O<sub>2</sub> saturation, including angiogenesis, glucose transport and erythropoiesis. Previous work has demonstrated that HIF1-alpha is modulated via the mTOR pathway and is sensitive to rapamycin inhibition. Clinical research has also verified that TS sufferers show high secretion levels of the HIF1-alpha target VEGF. HIF dysregulation may, therefore, play a significant role in the pathogenesis of TS patients. Increased HIF1-alpha activity will facilitate the formation and development of hamartomas by modulating the blood supply to increase the delivery of oxygen and nutrients to the core of the tumour. This makes it a potentially appealing therapeutic target for treatment of TS.

The mechanisms behind mTOR dependent HIF regulation have yet to be fully elucidated, therefore we have carried out a series of experiments to attempt to further clarify the pathway.

The use of various techniques including a luciferase reporter construct, kinase assays and quantitative-pcr have allowed us to confirm HIF1-alpha as a target of mTOR that is responsive to insulin stimulation but subject to rapamycin inhibition. Furthermore we have been able to eliminate the mTOR target S6K1 as a regulator of HIF1-alpha. We found HIF-1-alpha is regulated by 4E-BP1, however further analysis also suggests a potential secondary mechanism for HIF1-alpha inhibition which is dependent upon TSC2 but *independent* of the mTORC1 complex. Functional analysis of various patient derived mutants of TSC2 has also revealed the importance of the calmodulin binding domain (also referred to as the transcriptional activation domain) of TSC2 in regulating HIF1-alpha.

The regulation of HIF1-alpha is undoubtedly a complex and multi-faceted process. However further clarification of the pathway is fundamental to understanding the pathogenesis TS and therefore developing effective and targeted treatment strategies.

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## **MTOR AND ITS ASSOCIATED PHOSPHATASE SUBUNITS REGULATE STAT1 NUCLEAR TRAFFICKING AND THE INDUCTION OF STAT1-DEPENDENT GENES**

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Hamartomatous lesions in patients with tuberous sclerosis complex are characterized by dysregulated growth and excessive activity of 'mammalian target of rapamycin' (mTOR). mTOR is a highly conserved serine/threonine kinase that regulates protein translation, ribosomal biogenesis and cytokinesis. In *S. cerevisiae*, TOR, via its associated phosphatase subunits Tap42 and PPH22 or Sit4, also modifies gene transcription by suppressing the nuclear localization of stress response transcription factors (e.g., Msn2/4, Gln3). In mammalian cells, a physical association between mTOR and the transcription factor 'signal transducer and activator of transcription-1' (STAT1) was observed, the functional consequences of which were unknown. Because mTOR did not appear to be a kinase for STAT1, we hypothesized that similar to its homologue in yeast, mTOR regulates STAT1 nuclear localization, and the transcription of STAT1-dependent genes. By biochemical fractionation or confocal imaging of human lung epithelial adenocarcinoma (A549) cells, inactivation of mTOR with rapamycin enhanced STAT1 nuclear content in 'protein phosphatase 2A catalytic subunit' (PP2Ac)-dependent fashion. The increase in nuclear STAT1 levels was associated with enhanced synthesis of early (i.e., IRF-1, STAT1) and late (i.e., Caspase-1, Fas) pro-apoptotic genes in cells exposed to the cytokine IFN-gamma. Cells depleted of the Tap42 homolog alpha4 or PP2Ac exhibited enhanced expression of these IFN-gamma-stimulated tumour suppressor genes. mTOR physically associated with STAT1, alpha4, and PP2Ac in a dynamic macromolecular complex. Taken together, these results suggest that mTOR acts as an endogenous suppressor of STAT1-dependant gene expression by preventing STAT1 nuclear localization in PP2Ac/alpha4-dependant fashion. The mTOR/STAT1 signaling axis may represent an alternative target for the induction of growth arrest or apoptosis in TSC2-deficient lesions.

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# **TUBEROUS SCLEROSIS COMPLEX IN THE CLASSROOM; IMPLICATIONS FOR TEACHING AND LEARNING**

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Tuberous Sclerosis Complex (TSC) is a neurocutaneous genetic disorder that causes tumours to grow in multiple organ systems. The resulting tumours have many consequences in terms of health and intellectual functioning. Despite the potential for complex cognitive and social/behavioural difficulties, little research in TSC has focused on the educational implications of TSC. This study examined the educational impacts and specific academic impacts of TSC on children in Grades 1 through 8. In-depth, semi-structured interviews were conducted with 10 parents of children with TSC and 6 of their teachers or educational assistants. Information regarding the children's academic difficulties, effective interventions and programming, and overall effects of TSC in the classroom was sought. Specific academic deficits emerged in this study, such as receptive language difficulties that did not match expressive skills as well as memory and retention difficulties. Children in this study benefited from a variety of accommodations to their educational programming. Repetition and re-teaching of new material emerged as important considerations, as did use of visuals. Educators may benefit from more information about TSC, and may not be currently adequately prepared to accommodate and program for children with complex disorders such as TSC. Children with TSC who function in the cognitively average range may experience subtle differences that may require intervention and programming, and may benefit from testing such as psychological educational evaluations to identify subtle deficits, and ensure appropriate interventions are in place.

## **ABERRANT HYPERACTIVATION OF AKT AND MAMMALIAN TARGET OF RAPAMYCIN COMPLEX 1 (MTORC1) SIGNALING IN SPORADIC CHORDOMAS**

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Chordomas are rare, malignant bone neoplasms in which the pathogenic mechanisms remain unknown. Interestingly, Tuberous Sclerosis Complex (TSC) is the only syndrome where the incidence of chordomas has been described. We previously reported the pathogenic role of the TSC genes in TSC-associated chordomas. In this study, we investigated whether aberrant TSC/mTORC1 signaling pathway is associated with sporadic chordomas. We assessed the status of mTORC1 signaling in primary tumors/cell lines of sacral chordomas and further examined upstream of mTORC1 signaling, including PTEN (phosphatase and tensin homologue deleted on chromosome ten) tumor suppressor. We also tested the efficacy of the mTOR inhibitor rapamycin on signaling and growth of chordoma cell lines. Sporadic sacral chordoma tumors and cell lines examined commonly displayed hyperactivated Akt and mTORC1 signaling. Strikingly, expression of PTEN, a negative regulator of mTORC1 signaling, was not detected or significantly reduced in chordoma-derived cell lines and primary tumors. Furthermore, rapamycin inhibited mTORC1 activation and suppressed proliferation of chordoma-derived cell line. Our results suggest that loss of *PTEN* as well as other genetic alterations which result in constitutive activation of Akt/mTORC1 signaling may contribute to the development of sporadic chordomas. More importantly, a combination of Akt and mTORC1 inhibition may provide clinical benefits to chordoma patients.

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## NF2/MERLIN IS A NOVEL NEGATIVE REGULATOR OF MTOR COMPLEX 1 AND ACTIVATION OF MTORC1 IS ASSOCIATED WITH MENINGIOMA AND SCHWANNOMA GROWTH

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Inactivating mutations of the neurofibromatosis 2 gene, *NF2*, result in predominantly benign neurological tumors, schwannomas and meningiomas in humans, however, mutations in murine *Nf2* lead to a broad spectrum of cancerous tumors. The tumor suppressive function of the NF2 protein, merlin, a membrane-cytoskeletal linker, remains unclear. Here we identify the mammalian target of rapamycin complex 1 (mTORC1) as a novel mediator of merlin's tumor suppressor activity. Merlin-deficient human meningioma and merlin knockdown arachnoidal cells, the non-neoplastic cell counterpart of meningiomas, exhibit rapamycin sensitive constitutive mTORC1 activation and increased growth. NF2 patient tumors and *Nf2*-deficient MEFs, demonstrate elevated mTORC1 signaling. Conversely, exogenous expression of wild type merlin isoforms, but not a patient-derived mutant, L64P, suppresses mTORC1 signaling. Merlin does not regulate mTORC1 via the established PI3K-Akt or MAPK/ERK-mediated TSC2 inactivation, and may instead regulate TSC/mTOR signaling in a novel fashion. In conclusion, deregulation of mTORC1 signaling, which attenuates growth factor-induced Akt activation through negative feedback mechanisms, may limit the growth of human NF2-associated tumors and suggests that rapamycin may be therapeutic for NF2.

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## PHOSPHORYLATION OF TSC1 AT RESIDUE T417 RESULTS IN MITOCHONDRIAL LOCALIZATION AND HSP70-DEPENDENT REGULATION OF APOPTOSIS

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The products of the tuberous sclerosis complex (TSC) genes, TSC1 and TSC2, form a heterodimer that suppresses the kinase mammalian target of rapamycin (mTOR). Recently we reported that the TSC1/2 complex directly interacts with Hsp70. However, the molecular mechanisms underlying this interaction have not yet been clearly defined. Herein we demonstrate that the TSC1/2 complex and Hsp70 are both localized to the outer mitochondrial membrane. Moreover, localization of the TSC1/2 complex to the mitochondria requires the presence of Hsp70. In addition, a non-phosphorylated mutant form of TSC1, T417, which cannot bind to Hsp70, does not localize to the mitochondria. Increased expression of cleaved caspase-3 and PARP was observed in TSC1-deficient cells, indicative of enhanced apoptosis. This increase was suppressed by transfection of Myc-TSC1 (WT) and N-TSC1 (AA 1-511), but not by C-TSC1 (AA 512-1164). Furthermore, all non-phosphorylated TSC1 mutants (T357A, T390A) inhibited apoptosis in TSC1-deficient cells, whereas T417A resulted in increased apoptosis. We also observed that in the presence of KNK437-mediated inhibition of HSP70 expression, all non-phosphorylated TSC1 (T357A, T390A, T417A) did not inhibit apoptosis. Finally, we conclude that both phosphorylation of TSC1 at T417 and direct interaction with Hsp70 are required for mitochondrial localization and regulation of apoptosis.

## LEARNING AND MEMORY IN TSC2+/- (EKER) RATS CHALLENGED WITH EXPERIMENTAL EPILEPSY

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Tuberous Sclerosis Complex (TSC) is characterised by severe mental retardation in a part of the affected patients. Many TSC patients also suffer from epilepsy. The onset and severity of epilepsy, notably the occurrence of infantile spasms, is strongly associated with the development of mental retardation in TSC. The TSC2+/- (Eker) rat is an animal model of TSC showing impaired synaptic plasticity and alterations in episodic-like memory, however no obvious cognitive deficit. We asked here whether experimental epilepsy in TSC2+/- (Eker) rats would induce a deficiency in learning and memory resembling the association of TSC mutation, epilepsy and mental retardation observed in TSC patients. Male offspring from the TSC2+/- (Eker) rat line was challenged with status epilepticus induced by injections with kainic acid at days 7 and 14 after birth. At the age of 3-6 months, four experimental groups (wild-type naïve, wild-type with epilepsy, TSC2+/- (Eker) naïve, TSC2+/- (Eker) with epilepsy) were analysed for behavioural abnormalities. Rats which underwent epilepsy showed more anxiety in the light/dark-box, irrespective of genotype, as has been demonstrated previously for kainic acid-induced status epilepticus. No differences, however, could be observed between the four groups for explorative behaviour in the open field test, classical conditioning in the fear conditioning paradigm, spatial memory in the Morris water maze and recognition memory in the novel object recognition paradigm. Infantile spasms itself have been described as causes of mental retardation, also outside of TSC. An interpretation limited to our data only may suggest that it is rather the individual mutation in TSC or the individual severity of epilepsy, notably infantile spasms, which causes severe mental retardation in TSC, but not necessarily a combination of these two factors. Still, other animal models of TSC could describe a primary effect of TSC mutations on impaired learning and memory, irrespective of epilepsy. We therefore suggest that other TSC animal models, which showed deficits in learning and memory, should also be challenged with experimental epilepsy.

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## **CALMODULIN-DEPENDENT ALTERATION OF INTRACELLULAR LOCALIZATION OF TUMOR SUPPRESSOR PROTEINS (TSC1, TSC2)**

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TSC1 (Hamartin) and TSC2 (tuberin) form a heterocomplex and serve as a GTPase activating protein (GAP) against Ras homolog enriched in brain (Rheb). Rheb in turn regulates mammalian target of rapamycin (mTOR) and ribosomal S6 Kinase (S6K). We have previously reported that TSC1 and TSC2 are present in both the microsomal and cytosol cellular fractions, which led us to hypothesize that there is a TSC1/TSC2 releasing mechanism. Moreover, based upon the amino acid sequence, there is a calmodulin (CaM) binding site at the C-terminus of TSC2.

Here, we report that TSC2 was released from the membrane fraction in HeLa (human cervix adenocarcinoma) cells when the  $Ca^{2+}$  concentration was enhanced by tunicamycin treatment. This was dependent on activation of CaM, but TSC1 was not required for release. TSC2 and CaM did not maintain their complex after release from the membrane and further analysis revealed that TSC2 was transferred into the nucleus where it formed a complex with the vitamin D receptor (VDR).

Our data demonstrate for the first time that activation of CaM is essential for releasing TSC2 from the membrane and TSC2 may have a novel function in the nucleus via the VDR.

## ABNORMAL NEURAL DEVELOPMENT AND CELL SIZE DEFECTS IN A ZEBRAFISH MODEL OF TUBEROUS SCLEROSIS

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Tuberous Sclerosis Complex (TSC) is an autosomal dominant disease due to mutations in either the *TSC1* (encodes hamartin) or *TSC2* (encodes tuberin) genes. Patients with TSC have hamartomas in various organs throughout the whole body, most notably brain, skin, eye, heart, kidney and lung. To study the development of hamartomas, we generated a zebrafish model of TSC featuring a nonsense mutation in *tsc2* (*vu242*) gene. The resulting truncated form of tuberin lacks the GAP domain that is required for inhibition of Rheb and the TOR kinase within TORC1. We show that *tsc2*<sup>*vu242*</sup> is a recessive larval lethal mutation that causes increased cell size in the brain and liver. Greatly elevated TORC1 signaling is observed in *tsc2*<sup>*vu242/vu242*</sup> homozygous zebrafish with moderate increased signaling in *tsc2*<sup>*vu242/+*</sup> heterozygotes. Moreover, wild-type embryos overexpressing the truncated tuberin also have increased TORC1 activity indicating a dominant negative mode of action. Neurons are poorly organized in the forebrain of *tsc2* homozygous mutants with extensive grey/white matter disorganization by ectopically positioned neurons. We then generated zebrafish mosaic for the *tsc2*<sup>*vu242/vu242*</sup> mutation and found expected increased TORC1 signaling in a cell-autonomous manner but also found that mutant cells recruited wild-type cells to ectopic regions of the forebrain in a non-cell autonomous manner. These results demonstrate a highly conserved role of *tsc2* in zebrafish and establish a new animal model for novel studies of TSC. The finding of non cell-autonomous function of mutant cells may help explain the formation of brain tumors and cortical malformations in human TSC without requirement for a second mutation.

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## **PROTEIN DEGRADATION AND NEURONAL DEGENERATION IN BRAIN CORTEX OF TSC PATIENTS**

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Tuberous sclerosis complex (TSC) is an autosomal dominant tumor syndrome that affects approximately 1 in 6000 individuals and in which affected individuals develop mental retardation, developmental brain defects and seizures. One of the TSC gene products, tuberin is assumed to be the functional component being involved in a wide variety of different cellular processes. We have recently reported that tuberin was hyperphosphorylated in post-mortem frontal cortex tissue of both AD and PD/DLB patients and both, P-TEN and Akt phosphoactivation corresponded to the hyperphosphorylation patterns of tuberin suggesting that PTEN-Akt pathway might be the mechanism of tuberin phosphorylation in these disorders. In the present study, we show that brain cortex obtained from TSC patients have decrease parkin-mediated ubiquitination activity as compared to the cortex of age-matched control subjects. Although the levels of parkin were equal in control and patients samples, the E3 ligase activity of parkin as measured by ubiquitination was totally lost in the patient samples. In addition, total ubiquitin levels were also decreased in patients sample as compared to controls. Our data suggest that failure of parkin-dependent ubiquitin proteasome mediated protein degradation pathway might be a mechanism in the neurodegeneration process during TSC. (This work was supported in part by grants from the American Diabetes Association and New Investigator Award from South Texas Veterans Healthcare System (S.L.H.).

## DIFFERENTIAL REGULATION OF PROTEIN KINASES IN TSC-DEFICIENT RENAL ANGIOMYOLIPOMAS AND RENAL CELL CARCINOMA

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Tuberous sclerosis complex (TSC) is associated with benign human tumors in many organs, including angiomyolipomas in the kidneys and rarely associated with renal cell carcinoma (RCC). On the other hand, deficiency of TSC in Eker rat is associated with RCC. The tuberous sclerosis complex (TSC) is caused by defects in two tumor suppressor genes, *TSC-1* and *TSC-2*. We have recently reported that tuberin was totally lost in kidney tumor of Eker rats. In the present study, we investigated the distribution of phospho-serine/tyrosin kinases, PI3-K activity, PTEN, p-tuberin, tuberin, hamartin, p-mTOR and P-p70S6K in kidney tumor of Eker rats and in angiomyolipomas kidney tissue of TSC patients compared to control kidney tissues. Our data shows an increased in serine and tyrosin kinases in both angiomyolipoma and RCC tissues. Activity of PI3-K was only detected in RCC of Eker rats compared to renal angiomyolipoma tissues. In addition, PTEN was highly expressed in renal angiomyolipoma tissues while completely lost in kidney tissue with RCC of Eker rats. Phosphorylation of tuberin was significantly increased in both RCC and angiomyolipomas tissues compared to control tissues. However, total tuberin was significantly decreased in angiomyolipomas tissues while completely lost in kidney tumor of Eker rats. In addition, hamartin was highly expressed in both in angiomyolipoma tissues and kidney tumor of Eker rats. Activation of mTOR and phosphorylation p70S6K were also significantly increased in both RCC from Eker rats and renal angiomyolipoma tissues compared to control tissues. These data provide novel evidence that deficiency of both PTEN and tuberin but not hamartin are major tumor suppressor genes involve in development of RCC. (This work was supported in part by grants from the American Diabetes Association and New Investigator Award from South Texas Veterans Healthcare System (S.L.H.).

## **LYMPHANGIOLEIOMYOMATOSIS- EXPERIENCE WITH ANALYSIS AND CULTURE OF 27 CHYLOUS PLEURAL EFFUSIONS**

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Lymphangioleiomyomatosis (LAM) is a rare cystic disease with progressive lung destruction and development of small nodules containing smooth-muscle like cells and lymphatic channels. LAM occurs at high frequency in adult women with Tuberous Sclerosis Complex (TSC). It also occurs in women without TSC, so-called sporadic LAM. Sporadic LAM is typically more severe than TSC-associated LAM. Chylous pleural effusions are seen in about 20% of LAM patients.

We have had the opportunity to study thoracentesis fluids from 19 patients with sporadic LAM, a total of 27 samples as some patients have provided multiple samples. Chylous pleural fluids contain a variable proportion of LAM cell clusters, as originally described by Seyama et al. (Am J Surg Pathol. 2005 29:1356-66). In our experience 12 samples contained relatively abundant LAM clusters, 5 fluids had a few clusters, and 10 samples had no or minimal clusters. LAM cell clusters consist of a central core of smooth muscle like cells that express melanocytic markers (HMB45, MART-1) and a surface layer of lymphatic endothelial cells that are LYVE+ and VEGFR3+. Some pleural fluid samples (4 of 27) contained irregularly shaped groups of cells that were of a different morphology, not true LAM cell clusters, and of uncertain origin.

In culture, we obtain Short-Term-Cultured LAM cell clusters (STC-LCC) which consist of large elongated smooth muscle-like cells and nests of lymphatic endothelial cells (LEC). Smooth muscle media supports attachment of smooth muscle like cells (smooth muscle actin+, pS6(S240/244)+), but promotes growth of numerous smaller cells of myofibroblast type from these fluids, while LEC are suppressed. This observation argues against a hypothesis that LAM cells secrete VEGF-D that supports LEC survival and growth. On the other hand, in endothelial media with VEGF-A both large SMC-like cells and LEC cells attach and spread initially. However, in all conditions tested, there is no appreciable growth of the LAM cells (Ki67-), suggesting that current in vivo conditions do not match with the growth needs of LAM cells.

## EFFECTS OF A TSC2 HYPOMORPHIC ALLELE IN THE BRAIN

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Tuberous sclerosis complex (TSC) is a human genetic disease that causes a range of neurologic phenotypes, including developmental delay, mental retardation, and autism spectrum disorder. Recently two reports have described the finding of cognitive and functional deficits in mice heterozygous for a *Tsc1* or *Tsc2* mutation (*Tsc1*<sup>+/−</sup>, *Tsc2*<sup>+/−</sup> mice).

We have recently described a novel hypomorphic allele of *Tsc2* called *del3*, in which exon 3 encoding 37 amino acids near the N-terminus of tuberin is deleted (Hum Mol Genet. 2009 8:2378-87). This mutation leads to reduced but not absent expression of *Tsc2* with consequent changes in mTOR and AKT signaling pathways. *Tsc2*<sup>del3/del3</sup> embryos die at E13.5 (1-2 d later than *Tsc2*<sup>−/−</sup> embryos), while *Tsc2*<sup>del3/4</sup> mice have a milder tumor phenotype than *Tsc2*<sup>+/−</sup> mice. Both *Tsc2*<sup>del3/del3</sup> MEF lines and embryos show activation of mTORC1 and reduced pAKT-S473.

We have used a conditional form of this allele, denoted *c-del3*, to begin to examine the effects of reduced *Tsc2* expression in the brain. *Tsc2*<sup>c-del3/c-del3</sup> *syn1cre+* mice are born in Mendelian ratios, and appear to have normal survival out through 40d, and no apparent phenotype; in marked contrast to *Tsc1*<sup>cc</sup> *syn1cre+* mice (J Neurosci 2008 28:5422-32). *Tsc2*<sup>c-del3/c-del3</sup> *syn1cre+* mice show mildly reduced weight gain, but normal brain weight. Immunoblot analysis of brain lysates show a modest, 2-fold elevation in phospho-S6, both Ser235/236 and Ser240/244 sites, while pAkt-S473 levels appear normal. *Tsc2* levels in the brain are about 50% of those of controls. Pathological and histological studies are in progress.

To extend this allelic series, we have begun breeding to generate mice with the genotype *Tsc2*<sup>c-del3/−</sup> *syn1cre+*. In these mice, we expect that *Tsc2* protein levels will be one half lower than those seen in neurons in the *Tsc2*<sup>c-del3/c-del3</sup> *syn1cre+* mice, with a corresponding enhancement of phenotype. Planned studies in both sets of mice include brain histology with extended IHC, behavioral studies and signaling downstream TSC1/TSC2 complex.

We have also generated mice that have the genotype *Tsc2*<sup>c-del3/c-del3</sup> *nestin-cre+*. These mice have a median survival of 80 days, with significant brain enlargement due to bigger neurons in cortex and glial cells mostly in hippocampus, both coming from nestin-expressing progenitors. We found marked pS6 increase in brain lysates from the *Tsc2*<sup>c-del3/c-del3</sup> *nestin-cre+* mice, accompanied by reduced *Tsc2* and pAkt-S473. The longest surviving mice die at age 6 months, likely due to renal cystic disease and dehydration. They are generally less active than normal controls. In contrast *Tsc1*<sup>cc</sup> *nestin-cre+* mice uniformly die at birth, with enlarged brains and dramatic mTOR signaling changes.

# ESTROGEN ENHANCES RESISTANCE TO ANOIKIS IN TUBERIN-DEFICIENT CELLS

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## Introduction

Genetic evidence indicates that lymphangiomyomatosis (LAM) is the result of benign metastasis of tuberin (Tsc2)-null cells. The molecular mechanisms underlying LAM pathogenesis is not well known. Metastatic cancer cells have the ability to resist anoikis—apoptosis due to loss of extracellular matrix attachment. In a xenograft model of LAM, we found that 17-beta-estradiol ( $E_2$ ) causes a significant increase in circulating tumor cells and promotes lung metastasis. The metastatic phenotypes are associated with the activation of p42/44 MAPK and are inhibited by MEK1/2 inhibitor, CI-1040. We hypothesize that  $E_2$  promotes survival of Tsc2-null cells placed in circulation. To determine the components that mediate estrogen-enhanced survival of Tsc2-null cells, we analyze the pro-apoptotic protein Bim (Bcl-2 interacting mediator of cell death), a critical activator of anoikis. Bim is phosphorylated by p42/44 MAPK, leading to proteasomal-mediated degradation.

## Method

ELT3 cells (Eker rat uterine leiomyoma-derived smooth muscle cells) were cultured with or without 10 nM  $E_2$  in serum-free and phenol-red free medium supplemented with 10% charcoal-stripped FBS for 24 hours. Cells were harvested, and plated onto PolyHEMA dishes. Bim and cleaved caspase-3 protein levels were determined by immunoblot analysis. To further understand how Bim is regulated in Tsc2-null cells, cells were treated with proteasome inhibitor, MG132, and MEK1/2 inhibitor, PD98059. For adherent conditions, ELT3 cells were cultured with or without 10 nM  $E_2$  and/or 0.5  $\mu$ M MG132 or 50  $\mu$ M PD98059 in the same medium. DNA fragmentation was detected using Cell Death Detection ELISA kit.

## Results

We found that estrogen decreases levels of cleaved caspase-3 and DNA fragmentation, indicating that  $E_2$  promotes resistance to anoikis. We also found that Bim accumulation is reduced at 1 hour of detachment, which is associated with enhanced cell survival. PD98059 treatment blocks  $E_2$ -reduced Bim and increases levels of cleaved caspase-3 and DNA fragmentation, suggesting that  $E_2$ -induced resistance to anoikis is MAPK-dependent. In adherent cells we found that estrogen decreases Bim transcripts at 24 hours of  $E_2$  stimulation, measured by real-time RT-PCR.  $E_2$  also reduces Bim protein levels. Pre-incubation of cells with MG132 and PD98059 blocks estrogen's reduction of Bim. In conclusion, we found that  $E_2$  regulates pro-apoptotic protein Bim, in Tsc2-null cells. Inhibition of MEK1/2 and proteasomal activity block  $E_2$ -regulated Bim accumulation and cell survival. We anticipate that targeting signaling pathways contributing to Bim activation and proteasome activity may have clinical specificity and significance in treating LAM.

## EFFECTS OF CONDITIONALLY INACTIVATING *TSC1* IN THE THALAMUS DURING DEVELOPMENT IN A MOUSE MODEL OF TUBEROUS SCLEROSIS

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Tuberous Sclerosis (TS) is a complex genetic disorder caused by mutations in either the *Tsc1* or *Tsc2* gene. One major feature of TS is cognitive impairment, often involving autism spectrum disorders, epilepsy and mental retardation. The thalamus (Thal) is vital to cognition, as it serves a critical role in information processing and integration in the brain. Additionally, it has been found that the Thal shows a significant reduction of gray matter volume in TS brains, and this effect has been linked to poor performance in various memory tasks (Ridler 2007). Because TS onset can be observed perinatally, we hypothesize that perturbations of thalamic development may be an underlying factor in the cognitive deficits of TS. Thus, we have inactivated the *Tsc1* allele in the Thal at various embryonic stages and have analyzed the resulting phenotype at later embryonic or postnatal stages.

To temporally and spatially target *Tsc1* inactivation we utilize a conditional *Tsc1* mouse line (Kwiatkowski 2002) crossed with *Gbx2*<sup>CreER-IRES-eGFP</sup>; *R26R* mice (Chen 2009; Soriano 1999). The conditional *Tsc1* allele (*Tsc1*<sup>fl</sup>) contains *loxP* sites flanking exons 17 and 18. CreER recombinase, expressed under the control of a tissue-specific promoter (*Gbx2*), provides spatial (Thal) selectivity. The modified estrogen receptor (ER) domain of CreER keeps the protein in an inactive state until tamoxifen is administered, giving us temporal control over its activity. Once activated, CreER mediates recombination between the two *loxP* sites in the *Tsc1* gene and renders *Tsc1* inactive only in thalamic cells which express CreER. Simultaneously, we use the *R26R* reporter, which produces beta-galactosidase (Bgal) after recombination. Because the Bgal marker is expressed heritably, constitutively and permanently, we mark only cells in which *Tsc1* has been inactivated.

We induced *Gbx2*<sup>CreER</sup>-mediated recombination at three stages of development (embryonic day (E)12.5, E14.5, and E17.5) and analyzed mice at E14.5 and E17.5 to assess the acute phenotype, or at postnatal day (P)30 to determine the long-term effects of *Tsc1* inactivation during embryogenesis. We compared the loss of one or both copies of *Tsc1* versus wild-type controls and assessed the phosphorylation of S6 protein (p-S6), a downstream protein of the TSC/mammalian target of rapamycin (mTOR) pathway, which is used as a readout of *Tsc1* loss of function. Embryos with *Tsc1* inactivated at E12.5 show substantial increases in p-S6 levels which negatively correlate with the number of functional *Tsc1* alleles at E14.5 or E17.5. Samples with *Tsc1* inactivated at E14.5 demonstrate an increase in soma size, as well as greatly increased p-S6 levels as compared to controls at P30. In preliminary experiments using an independent Cre driver (*R26R*<sup>CreER</sup>), we have observed changes in innervation of the frontal cortex, suggesting possible perturbation of thalamocortical circuitry. Our future directions include further analysis of alterations in thalamocortical projection patterns and the use of thalamic electrophysiological recordings in thalamocortical slices of wild-type and *Tsc1*-mutant pups to analyze functional changes in response to *in vivo* loss of *Tsc1* function.

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## **AN AUSTRALIAN TSC COHORT: ARE SURVEILLANCE GUIDELINES BEING MET?**

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**Aims:** The aims of this study were to describe the clinical and radiological features of 45 Australian patients with Tuberous Sclerosis Complex (TSC), to assess genotype-phenotype correlation and to evaluate adherence to surveillance guidelines.

**Methods:** Children and adults who fulfilled criteria for a diagnosis of TSC were recruited. TSC1 / TSC2 gene sequencing and MLPA were performed. Clinical and radiological features and recent surveillance were recorded.

**Results:** There were 45 patients (22 males, 23 females) of whom 31 were children (age 0-17) and 14 adults (age >18). 39 patients had de novo TSC, 5 were familial and 1 was uncertain. There were 9 (20%) patients with TSC1 mutations, 22 (49%) with TSC2 mutations, 4 (9%) unclassified variants (UCV), and in 10 patients (22%) no mutation was identified (NMI).

90% of patients had hypomelanotic macules, 78% of patients had seizures, 60% developmental delay and 32% autistic spectrum disorder. Cortical tubers were present in 88% of patients, renal cysts in 37% and angiomyolipomata in 39%.

10/22 (45%) patients with TSC2 mutations had autistic spectrum disorder (ASD), compared with 1/9 (11%) TSC1 patients and 1/10 (10%) NMI patients ( $p < 0.05$ ). While trends suggesting increased severity with TSC2 mutations for other clinical features were observed, these did not reach statistical significance.

33/45 (73%) patients were undergoing recommended surveillance. 28/ 31 (90 %) of paediatric patients met surveillance guidelines, compared with 5/14 (36%) adult patients ( $p < 0.05$ ).

**Discussion:** The mutation and phenotypic spectrum is consistent with previous studies. Patients with TSC2 mutations had a significantly increased risk of ASD compared with those who had TSC1 mutations and those who were mutation negative. Most adults in our study were not meeting surveillance guidelines.

## IDENTIFICATION OF TSC2 SECOND HIT MUTATIONS IN TSC BRAIN TUBERS

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Supported by the TS Alliance and NIH NINDS.

**Introduction:** Tubers are an extremely important aspect of the TSC phenotype, and their pathogenesis remains uncertain, due to a lack of confirmation as to whether they occur due to second hit events in TSC1/TSC2 or an alternative pathogenetic mechanism.

**Methods:** Tuber samples were obtained from the U Maryland Brain and Tissue Bank (18) or from U. Cincinnati (14). Sequence analysis was performed using the ultra-deep pyrosequencing (UDPS) technique of 454 Sequencing on the Genome Sequencer FLX system (Roche). Sequence variants were confirmed by SNaPshot. Functional analysis was performed in HEK293 cells.

**Results:** Two second hit TSC2 mutations were identified in the analysis of 32 tubers. In one post-mortem sample, the subject had a germline TSC2 4375C>T R1459X nonsense mutation. In 7 separate tuber samples from this patient, the 1864C>T R622W TSC2 missense variant was identified at levels from 0.7 to 7.2% by 454 sequencing. To confirm the 1864C>T variant, a SnaPshot assay was developed to quantify the level of mosaicism. We found that although a T signal was seen in control blood DNA samples, the signal was quite uniform at about 4.0%. In the 7 brain samples, the T allele signal ranged from 7.3% to 16.6% by SnaPshot, significantly higher than levels seen in the controls ( $p=0.0025$ ). For further confirmation, another 20 samples from this patient's brain were studied, and mutant T allele frequencies ranged from 4.0% to 18.4%, again a highly statistically significant difference in comparison to controls ( $p=0.0008$ ). Moreover, mutant allele frequency correlated roughly with per cent giant cells in these brain regions. Since the TSC2-R622W variant has not been previously characterized, we performed functional studies. In HEK293 expression experiments, TSC2-R622W did not bind to TSC1, in contrast to wild type TSC2. In addition, TSC2-R622W had reduced GAP activity in comparison to wild type TSC2 in HEK293 cells. In aggregate, these data suggest that in this patient a second hit TSC2 1864C>T mutation occurred early during brain development that resulted in its wide dissemination throughout the L cerebral hemisphere. The second apparent second-hit mutation we identified is a TSC2 4375C>T R1495X nonsense mutation in another patient. It was detected at 6.3% frequency in tuber DNA by 454 sequencing, and SnaPshot analysis demonstrated that in the control samples, there was no significant signal from the 4375C>T variant, while this was clearly seen in the patient's tuber DNA sample at a frequency of 4.2%. This patient was known to have a germline deletion in one allele of TSC2 (extending from 5' to TSC2 through exon 29), which was confirmed by MLPA.

**Conclusion:** In summary, we have identified two low frequency mutations in TSC brain tuber samples, which appear to have occurred as second-hit events during brain development. However, only 2 second-hits (one multiple times) were detected in 32 TSC brain tubers. This low frequency suggests that small point mutations in TSC1/TSC2 are uncommon in TSC tubers. Alternative pathogenetic mechanisms may apply in some tubers. However, it is likely that tuber second hit events are commonly due to large

genomic deletions, which cannot be detected by 454 Sequencing of amplicons targeting TSC exons.

## IDENTIFICATION OF MOSAICISM IN TSC BY NEXT GENERATION SEQUENCING

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Supported by the TS Alliance and NIH NINDS.

**Introduction:** Tuberous sclerosis complex (TSC) is an autosomal dominant neurocutaneous syndrome caused by mutations in *TSC1* and *TSC2*. Comprehensive mutation detection studies have led to identification of mutations in 85 - 90% of TSC patients. However, 10 to 15% patients have no mutation identified (NMI). Identification of mosaic mutations in NMI patients would be helpful both to confirm the clinical diagnosis, and to provide important information for genetic counseling and family planning.

**Methods:** We used the ultra-deep pyrosequencing (UDPS) technique of 454 Sequencing on the Genome Sequencer FLX system (Roche) to search for mosaicism in 40 TSC patients thought to have no *TSC1* or *TSC2* mutation by conventional methods. Allele specific PCR, SNaPshot, SURVEYOR analysis, and DHPLC were used to confirm mosaic mutations detected by 454.

**Results:** Multiple sequence variants were detected in the 454 analysis. Two reported *TSC2* mutations were identified, each at 5.3% read frequency in different patients, consistent with mosaicism. They were a splice site mutation (1444-1G>A) and a missense mutation (5228G>A).

Both mosaic mutations were confirmed by multiple methods, including allele-specific PCR, SURVEYOR digestion, DHPLC analysis, and SNaPshot sequencing. For the 5228G>A mutation, Snapshot showed that the mutation was present at 10.5% frequency in patient blood DNA, while blood DNA from his parents showed no signal. For the 1444-1G>A mutation, a mutant signal was detected in both patient (12.8%) and control (6.1%) DNA, but was significantly higher in multiple replicates from the patient ( $p=0.0001$ ).

Seven of 40 samples were found to have heterozygous non-mosaic mutations, missed by conventional techniques, in *TSC1* or *TSC2*.

**Conclusions:** The 454 sequencing approach permits detection of mosaic mutations that would not be detected by conventional sequencing due to low signal. However, we identified mosaic mutations in only 2 (6%) of 33 samples from TSC patients that did not have a mutation detected by conventional means. This low frequency suggests that the majority of TSC patients who have no mutation identified are not due to mosaicism, but rather other causes, which remain to be determined. Possibilities include very low level mosaicism or complete absence (<2%) of the mutation in blood DNA, missed mutations occurring in introns or other regions, and the existence of a third TSC gene.

## SEIZURES IN TUBEROUS SCLEROSIS COMPLEX: DIFFERENCES BETWEEN IN PATIENTS WITH TSC 1 OR TSC2 GENOTYPES

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**Introduction:** Two gene loci have been identified in TSC, TSC 1 is located on chromosome 1 and TSC 2 is located on chromosome 16. The aim of this study is to determine if there are any differences in the seizure characteristics and degrees of intellectual disability in those patients who were identified as having the TSC 1 gene and those having the TSC 2 gene. Patients with TSC in whom no gene was found or in whom genetic analysis was impossible were also included in the study.

**Method:** Patients with TSC, born after 1975 were identified using the data base kept at the regional TSC clinic. Information on the patient's seizures and any degree of intellectual impairment was obtained from the medical records in the department of medical genetics, other hospital notes, the patient's consultant who monitors the epilepsy and from the patient or their family. From these sources the following information was obtained: 1) Presence or absence of epilepsy 2) Age of seizure onset. 3) Type of presenting seizure 4) Degree of intellectual disability. 5) Type of presenting seizure 6) No of different seizure types

**Results:** Fifty five patients with TSC, born after 1<sup>st</sup> January 1975, were identified from the data base. Of these 4 were deceased. There were 27 females and 28 males. Age range was from 1 year 6 months to 32 years 9 months. The mean age was 17 years 7 months. Fifty patients (91%) had a history of epileptic seizures. In the 5 patients with no seizures 2 had the TSC1 gene, in 2 the analysis was not done and in 1 case no gene was identified during analysis. No intellectual disability was found in these patients. In patients with epilepsy 6 had the TSC 1 gene, 27 the TSC 2 gene and in 17 the status was unknown. Of these 17, in 12 patients the test was refused, 3 were deceased and in 2 the gene was not detected by analysis. In the 6 patients with the TSC1 gene, 3 had the seizure onset at less than one year, 1 had seizures after age of 5 and in 2 the information was not found. Mean age of onset was 3.91 years. Three patients presented with generalised seizures, 1 had complex partial seizures and in 2 cases the presenting seizure type was unknown. One patient had only 1 seizure type, three had more than 1 and in 2 it could not be determined. Of these 6 patients 3 had severe intellectual disability, 1 had moderate and 2 had none. In the 27 patients with the TSC 2 gene 19 had seizures starting before 1 year of age, 7 between 1 and 5 years and in 1 patient the information was not found. Mean age of seizure onset was 0.80 years. Thirteen patients with TSC 2 gene presented with infantile spasms, 4 with generalised seizures, 3 with absences, 3 with partial seizures and 1 with atonic seizures. In 2 cases the presenting seizure type was unknown and in 1 case the patient had multiple seizure types and was described as having polymorphous epilepsy. Eight had only 1 seizure type, 17 had more than 1 and in 2 it could not be determined. Of these patients 14 had severe intellectual disability, 8 had moderate and 5 had none. In the 17 patients in whom no gene was identified, 13 had seizures before one year of age and 4 had seizures between 1 and 5 years. Mean age of onset was 0.74 years. Nine patients presented with infantile spasms, 4 with partial seizures, 2 with generalised seizures, 1 with myoclonic seizures and 1 with absences. Five patients had only 1

seizure type and 12 had more than one. Of these 14 had severe intellectual disability, 1 had moderate and 2 had none. Statistical analysis between the occurrences of epilepsy showed no significant difference between the TSC1 patients and the TSC2 patients. There was a significant difference between the two groups in the age of onset of the seizures.

**Conclusions:** Through the TSC clinic database most of the patients in Northern Ireland with TSC born after 1975 were identified. Mutations in the TSC 2 gene were more frequent. The incidence of epilepsy and intellectual disability found are the same as in other studies. Although there were only 6 patients in the TSC1 group there are significant differences between that group and the TSC2 patients.

Epilepsy occurs in both genotypes and there is no significant difference between the two groups.

The age of onset of seizures is significantly lower in the TSC 2 patients. More patients in the TSC2 group presented with infantile spasms. In the TSC2 group 63% of patients had more than one seizure type with only 50% in the TSC 1 group. In each of the two groups there was no difference in the percentage of severe intellectual disability (50%) in the two groups. In the future it is hoped to have mutation analysis on some of the 17 patients in whom the mutation is not determined. The low mean age of seizure onset and the incidence of infantile spasms would suggest that there could be more TSC2 mutations.

Overall it is concluded that epilepsy is found in both genotypes but is more severe in TSC 2 mutations.

# **A RARE CASE: LONG TERM COURSE OF A GIRL WITH DOUBLE PHAKOMATOSIS, TUBEROUS SCLEROSIS AND NEUROFIBROMATOSIS TYPE 1 - EFFECTS OF RAPAMYCIN TREATMENT ON ANGIOMYOLIPOMAS AND EPILEPSY**

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## **Introduction:**

The rare case of a 13 ½ year old girl with tuberous sclerosis (TSC) together with neurofibromatosis type 1 (NF1) is presented. The clinical diagnosis of TSC is based on characteristic skin, brain, and kidney lesions. The diagnosis NF1 was suspected because of an increasing number of café-au-lait spots and a positive family history. Molecular analysis revealed a de novo TSC2 mutation, and a NF1 mutation inherited from the mother.

## **Clinical description:**

Tuberous sclerosis was already diagnosed shortly after birth on the basis of cardiac rhabdomyomas. Neurofibromatosis was suspected at an age of 2 with the presence of café-au-lait macules. Both diagnoses have been verified by molecular diagnostics at an age of 12. Most of the medical problems are caused by TSC: a therapy-resistant epilepsy, beginning with 2 months with infantile spasms, a giant-cell astrocytoma at an age of 3 years, which was resected at an age of 6 ½ years, and renal angiomyolipomas at an age of nearly 12 years. The patient had huge angiomyolipomas in the left kidney and was treated with the mTOR-inhibitor sirolimus, before enucleation of the largest lipomas at 13 years. During the 6 months of treatment with sirolimus, the antiepileptic drug therapy (AED) remained unchanged, and the effects of sirolimus on the epilepsy were studied. – In January 2008, treatment with sirolimus started with 4 mg twice daily for 3 days, and was then reduced to 2 mg/d. The AEDs vigabatrin 3500 mg and valproate 1500 mg were not changed, mesuximide was raised up from 450 mg to 600 mg daily during a stop of sirolimus on account of fungal skin infection and seizure aggravation, but finally reduced again to 450 mg/d. Initially the blood levels of sirolimus and the AEDs were controlled weekly, then during greater intervals. Sirolimus was adjusted to a maximal dosage of 5 mg/d in order to achieve a blood level of > 4 ng/ml. The EEG was controlled before, 2 and 5 months after beginning, as well as 1½ and 6 months after termination of the sirolimus therapy. Cranial and renal MRI were carried out before and 6 months after beginning of the therapy, renal MRI additionally 3 months, and cranial MRI 7 months after withdrawal of sirolimus. The seizures and the well-being were observed continuously.

## **Results:**

During sirolimus treatment and unchanged doses of AEDs, the blood levels of valproate and vigabatrin decreased, while the level of mesuximide remained more stable. Seizure frequency diminished, and freedom from seizures occurred after 4½ months of sirolimus therapy. The motor function of the left arm as well as the verbal understanding of the girl improved, but her mood changed, she was calmer, non-reactive, almost apathetic, stuporous. The EEG showed no longer a focal status over the frontal region, and an important reduction of multifocal spikes was observed. The blood levels of the AEDs were not changed. The diagnosis of a “forced normalization”, an “alternative psychosis” was made. Mesuximide was reduced from 600 to 450 mg/d. Thereby seizures

reappeared regularly and the child was normal again. MRI of the kidneys in June 2008 showed a marked reduction of the size of the angioliipomas. Sirolimus therapy was stopped in July 2008. – After surgical enucleation of the greatest, left sided angioliipomas in August 2008, the seizure situation remained stable under the same AEDs for the next months until April 2009, although the cerebral MRIs, done in 2008 and 2009, remained unchanged compared to those of 2007, i.e. showed no improvement. – The facial angiofibromas of the patient also showed no regression under sirolimus, in contrast even worsened, and had to be treated by surgical resection at the end of 2008.

Conclusion:

In our patient with simultaneous tuberous sclerosis complex and neurofibromatosis, treated with sirolimus because of renal angiomyolipomas, we observed a beneficial effect of sirolimus not only on the angiomyolipoma size but also on epilepsy, whereas cerebral tubers and facial angiofibromas remained unchanged. Despite reduced blood levels, freedom from seizures appeared in our case with unchanged AEDs, concurrent with reduced SW-activity in the EEG. – The question is, if in desperate particular cases of tuberous sclerosis a sirolimus therapy is justified for seizure reduction over a certain time, e.g. 6 months, to achieve a benefit and a long lasting effect for some more months - like corticosteroids in infantile spasms.