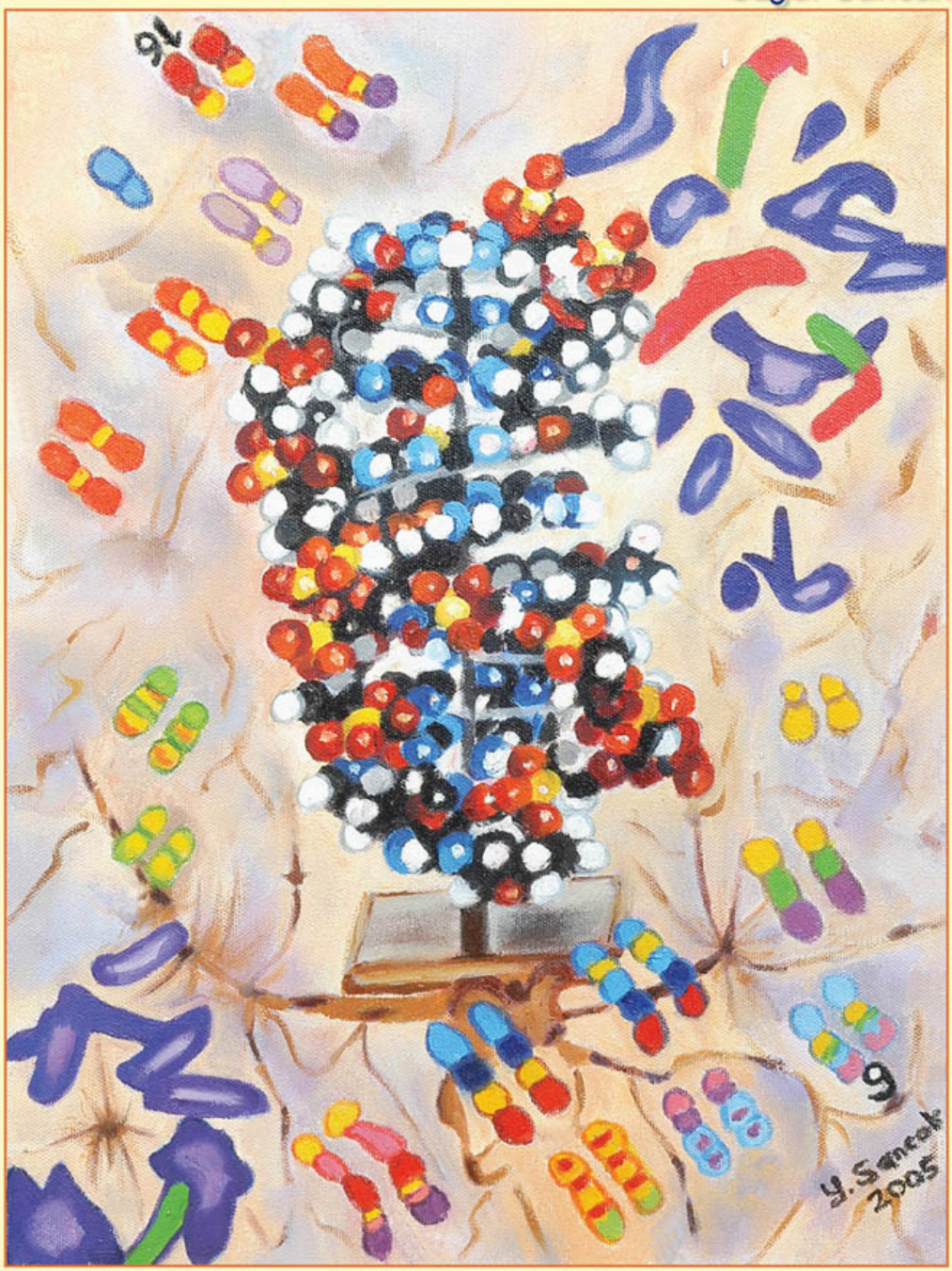
Tuberous Sclerosis Complex
Mutations, Functions and Phenotypes

Özgür Sancak



Tuberous Sclerosis Complex

Mutations, Functions and Phenotypes

Özgür Sancak

ISBN-10: 9090201939
ISBN-13: 9789090201931

The work presented in this thesis was performed at the Department of Clinical Genetics, Erasmus MC, Rotterdam, the Netherlands. The publication of this thesis was sponsored by the Stichting Tubereuze Sclerose Nederland.

Printed by PrintPartners Ipskamp, Enschede
Lay-out: Tom de Vries Lentsch
Cover painting: Yıldız Sancak

Tuberous Sclerosis Complex
Mutations, Functions and Phenotypes

Tubereuze Sclerose Complex
Mutaties, functies en fenotypen

Tüberoz Sklerozis Kompleks
Mutasyonlar, Fonksiyonlar ve Fenotipler

Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof.dr. S.W.J. Lamberts
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 14 december 2005 om 13:45 uur

door

Özgür Sancak

geboren te Elazig, Turkije

Promotiecommissie:

Promotor	Prof.dr. B.A. Oostra
Overige leden	Prof.dr. D. Lindhout Prof.dr. C.I. de Zeeuw Dr. A.J.J. Reuser
Copromotor	Dr. D.J.J. Halley

Sevgili anne ve babama

TABLE OF CONTENTS

3. DISCUSSION

1

INTRODUCTION

1. INTRODUCTION

1.a. Historical Overview

Tuberous Sclerosis Complex (TSC, OMIM #191100) is an autosomal dominant, multi-system disorder, first described with neurological and dermatological signs and symptoms.

Pierre François Olive Rayer published an atlas of skin diseases in 1835 [1], depicting a young man with small, erythematous papules in characteristic distribution on his face and with similar appearance to what Balzer and Ménétrier reported as *adenomas sébacés* (adenoma sebaceum) 50 years later [2], lesions closely resembling the facial angiofibromas often seen in patients with TSC.

In 1862, Friedrich Daniel von Recklinghausen presented a newborn infant who had died shortly after birth with cardiac tumors labeled as “myomata” and added briefly that the infant’s brain contained “great number of scleroses” [3]. This brief report contained the first description of two major pathologic lesions in newborn infants with TSC, as we now call them, cardiac rhabdomyomas and cortical tubers.

Désiré-Magloire Bourneville (1840-1909) gave the name still in use – *tuberous sclerosis of the cerebral convolutions* – to the cerebral pathology [4]. He described a 15-year-old female patient with first partial, and then generalized seizures accompanied by frequent episodes of status epilepticus and right spastic hemiplegia. The patient had skin tags (molluscum pendulum) of the neck and “confluent vesiculo-papular eruption on her nose, cheeks and forehead”. Pathologic examination of the brain disclosed raised, opaque, and sclerotic areas in some of the cerebral convolutions. Bourneville used the term *tuberous sclerosis of the cerebral convolutions* for the white nodular tumors embedded in the corpus striatum and protruding into the lateral ventricles he found on sectioning the brain sagitally.

A year later Bourneville and Brissaud reported another patient with the disease, a 4-year-old patient with right-sided seizures who died in status epilepticus and had similar cerebral pathology [5].

Hartdegen reported a 2-day-old infant who had died in status epilepticus. The case had spina bifida, purulent meningitis, areas of sclerosis throughout the cerebral cortex, and several small tumors protruding into the lateral ventricles of the brain containing hyperplastic glia and large cells that looked like neurons. Hartdegen called the cerebral lesion “glioma gangliocellulare cerebri congenitum” and suggested a neoplastic etiology which was also supported by Vogt and Bielschowsky later [6, 7]. In the mean time, dermatologists in France and Great Britain recognized and named “adenoma sebaceum” accompanied by seizures and mental retardation in some patients [2, 8].

In 1905 Campbell described ocular pathology [9] while in 1908, Vogt suggested the well-known

clinical triad of seizures, mental retardation and “adenoma sebaceum” as diagnostic for cerebral tuberous sclerosis [6]. Vogt also noted the cardiac and renal tumors as parts of the disease.
Pellizzi studied the cerebral histopatologies associated with the disease, emphasizing the dysplastic nature of the cerebral lesions [10]. He noted the disordered cortical architecture, embryonic appearance of the abnormal cells, neuronal heterotopia, and defective myelination. In 1905, Perusini followed by publishing a report on the microscopy of the cortical tubers including atypical neurons, subcortical areas of hypomyelination, and subependymal nodules also noting the association of cerebral, renal, and cardiac lesions with facial angiofibromas [11].

Kirpicznik [12], Berg [13], and Schuster [14] reported on the hereditary nature of the disease. The latter also described a case with only “adenoma sebaceum” and called this phenotype *forme fruste*, which was used as an indication of reduced phenotypic expression of the disease.

In 1920, van der Hoeve [15] noted the well-circumscribed lesions, the similarities between TSC, neurofibromatosis and von Hippel-Lindau disease in the patchy distribution of these lesions and their tendency to grow as benign tumors and introduced the terms *phakoma* and *phakomatosis*.

In 1924 Marcus [16] and in 1935 Dalsgaard-Nielsen [17] described intracranial calcifications as a clinical sign. With the introduction of pneumoencephalography, the intraventricular subependymal nodules on the walls of lateral ventricles could be demonstrated for the first time in 1934 [18].

In 1932, Critchley and Earl published the very first cohort, a cohort of 29 cases, with very detailed clinical descriptions and for the first time emphasized the diagnostic value of hypomelanotic macules as well as the presence of psychiatric disorders and autism [19].

Moolten, in 1942, renamed the disease as “the Tuberous Sclerosis Complex” with the appreciation of the complexity and the multiple organ involvement in this disorder [20].

In 1967, in a series of 71 TSC cases, 38% were found to have no mental retardation forcing the clinicians to question the validity of the Vogt triad as diagnostic criteria [21]. In the same report, a striking correlation was found between the presence of epilepsy and mental retardation.

With the improvement of imaging technology, including computed tomography, echocardiography, renal ultrasound and magnetic resonance imaging, came new and more extensive diagnostic criteria for diagnosis of TSC [22]. The estimated prevalence of TSC raised from 1:100,000 in the first half of the 20th century to 1:10,000 [23] and even to 1:5,800 [24]. Among the newly recognized cases were some with few or no clinical features of the previously used diagnostic criteria, the Vogt triad.

Gomez was the first person to describe the full clinical spectrum of TSC. In 1979, he published a monogram attracting the interest of both clinicians and genetic researchers to the field [25]. In 1992 and 1993 new guidelines for the diagnosis of TSC patients were established and definitive and presumptive TSC diagnosis classification was introduced [26, 27]. According to these

diagnostic criteria, facial angiofibromas, multiple ungual fibromas, cortical tubers, subependymal nodules, subependymal giant cell astrocytomas and multiple retinal astrocytomas were accepted as pathognomonic manifestations of TSC. In addition, having an affected family member was one of the diagnostic criteria.

In 1998 the diagnostic criteria for TSC have been revised and have taken its present form [28]. In its current form, no clinical sign or symptom is accepted as pathognomonic for TSC, and having an affected family member is no longer a diagnostic criterion. The present day diagnostic criteria are discussed in the following paragraphs.

1.b. Clinical Manifestations and Diagnostic Criteria of Tuberous Sclerosis Complex

Tuberous Sclerosis Complex is characterized mainly by the presence of three types of lesions proposed by Moolten [20]. *Hamartias* are well-circumscribed, misaligned or misarranged groups of dysplastic cells that nevertheless are appropriate for the organ or tissue involved. These undifferentiated cells do not multiply or grow more rapidly than the normal cells of the affected organ. *Hamartomas*, on the other hand, have a propensity to multiply excessively, growing as benign tumors. *Hamartoblastomas* are rare malignant tumors derived from hamartomas.

The phenotype of patients with TSC depends on the organ or organs involved, the number and size of the lesions as well as the location of the lesions. While some lesions do not appear until a certain age (i.e., angiomyolipomas), others may disappear with age (i.e., rhabdomyomas). The phenotypic heterogeneity of TSC is well documented, even within families [29-31].

The five main characteristics of TSC are (1) autosomal dominant inheritance; (2) age-dependent phenotypic expression (table 1); (3) characteristic lesions visible directly or indirectly; (4) lesions that are often multiple in the affected organ(s); and (5) well-circumscribed (benign) lesions in the affected organ(s) surrounded by normal parenchyma, except where a hamartoma causes mechanical displacement or compression.

Characteristic Lesions, Signs and Symptoms of TSC

Central Nervous System

Cortical tubers, subependymal nodules (SENs), subependymal giant cell astrocytomas (SEGAs), radial migration lines in cerebral white matter are the main signs of TSC in the central nervous system. Epilepsy and mental retardation are the most severe and incapacitating manifestations of TSC also associated with the expression of TSC in the central nervous system. Cranial imaging is recommended when a diagnosis of TSC is made or suspected. Neurological disorders in TSC are proposed to result from defects in cell migration, proliferation and differentiation. An abnormal population of neuroepithelial cells generated by the germinal matrix of TSC patients forms primitive, poorly differentiated cells expressing both neuronal and astrocytic features. Some of these cells remain in the germinal matrix zone leading to SENs and SEGAs, some migrate partially forming heterotopia in the subcortical white matter, while yet others migrate to the cortex forming the cortical tubers. Cortical tubers and SENs are present in 90-100% of the TSC patients.

SEGAs, although rarely seen in the general population (1.4%), is seen in approximately 15% of TSC patients. Although benign, these tumors can be locally invasive leading to progressive neurological dysfunction. SEGAs are almost always located near the foramen of Monro and therefore result in symptoms related to increased intracranial pressure. Herniation due to increased intracranial pressure may result in sudden deaths. Epilepsy is proposed to be caused by the architectural disturbances in the brain, the mass effect of the lesions or the increased excitability of some cell types typical to TSC lesions. Epilepsy is the most common neurological symptom of TSC, occurring in almost all TSC patients. In some patients, mapped epileptogenic lesions using a combination of electroencephalogram (EEG) and magnetic resonance imaging (MRI) data can be removed successfully. IQs of TSC patients are bimodally distributed, one group with severe mental disability and one with normal intelligence, although even the ones with normal intelligence have slightly lower IQ scores compared to their siblings. The degree of mental disability has been shown to be associated with the age of onset of seizures, presence of infantile spasms, and the number and localization of tubers. In addition to epilepsy and mental disability multiple behavioral problems including sleep disorders, hyperactivity, attention deficit, aggressiveness and autism have been reported in children with TSC. Good seizure control early in life is believed to reduce the mental and behavioral disabilities.

Skin

Skin manifestations of TSC can include hypomelanotic macules, shagreen patches, facial angiofibromas, fibrous forehead plaques and ungual (periungual or subungual) fibromas. Hypomelanotic macules are one of the most common lesions observed in TSC patients. 97% of the TSC patients have hypomelanotic macules compared to 4.7% in the general population. These lesions have normal number of melanocytes but show reduced number, melanization, and size of the melanosomes within the melanocytes and keratinocytes. Facial angiofibromas result from dermal fibrosis and vasodilation seen in approximately 75% of TSC patients. Fibrous forehead plaques, on the other hand, are fibromas without the vascular component. Shagreen patches, reported in 21-80% of the TSC patients depending on the age of the patients studied, are connective tissue hamartomas without increased vasculature, mainly located on lumbosacral region. Ungual fibromas, reported in 15-55% of TSC patients, are fibromas or angiofibromas histopathologically resembling facial angiofibromas or fibrous forehead plaques. Skin manifestations of TSC mainly raise cosmetic concerns and are treated accordingly.

Eyes

Astrocytic retinal hamartoma, also called retinal phakoma, is often located in the periphery of the retina and hence is mostly asymptomatic. They are seen in up to half the cases and can be bilateral in TSC patients. Other ophthalmologic lesions reported in association with TSC are retinal pigment alterations, strabismus, coloboma of the iris and angiofibromas of the eyelids. Most retinal lesions can be seen with direct or indirect fundoscopy and require no treatment.

Kidneys

After the central nervous system and skin, kidneys are the third most commonly involved organs in patients with TSC. Eventually, more than 80% of TSC patients will be found to have renal

involvement. Renal angiomyolipomas (AMLs) and multiple kidney cysts constitute the main manifestations in kidneys, although clear cell carcinomas can rarely be present. In a small proportion of TSC patients, adult type autosomal dominant polycystic kidney disease (ADPKD) is also present as a part of contiguous gene syndrome caused by the deletion of the *TSC2* gene and the neighboring PKD1 gene [32]. Renal AMLs, either as a sporadic condition or as a TSC manifestation, are more frequent among female patients. Although mostly benign, malignant AML with invasive growth may occur in TSC patients. Up to 80% of TSC patients might harbor renal AMLs, although the condition is not uncommon in the general population and is found in up to 2.1% of post-mortem examinations and only 2% of all AMLs are actual TSC patients. Kidney cysts observed in TSC patients have hypertrophic and hyperplastic cells lining the cyst wall, a distinct feature associated with only TSC. The renal manifestations of TSC may lead to hypertension, flank pain, hemorrhage and renal failure. The treatment of these lesions is mainly angiographic embolization for symptomatic lesions but may require surgical intervention including complete nephrectomy.

Heart

Cardiac rhabdomyomas are the most common congenital cardiac tumors, the majority of which are associated with TSC [33]. This condition can be the earliest sign of TSC *in utero*. 50-80% of the cases with multiple cardiac rhabdomyomas are associated with TSC. They are noninvasive, mitotically inactive and non-metastasizing overgrowths of cardiac muscle. Rhabdomyoma cells have numerous vacuoles filled with glycogen. They are large cells with small central nuclei and a thin peripheral rim of cytoplasm and are called “spider cells” for the delicate radiating strands connecting the nucleus with the cell membrane. Although they are benign and mostly regress during the first year of life, cardiac rhabdomyomas may severely compromise blood flow, interfere with myocardial function, and cause arrhythmias, and might lead to stillbirth or sudden death. Symptomatic cases where the tumor does not regress may require surgical intervention.

Lungs

Lymphangioleiomyomatosis (LAM), the lung manifestation of TSC, occurs almost solely in female TSC cases above 20 years of age. Recent reports indicate that LAM has a prevalence as high as 39% in TSC patients [34-36]. Pathologically it is characterized by the abnormal proliferation of immature smooth muscle-like cells that grow aberrantly in the lung. However, LAM is not necessarily limited to the lung but can also involve lymph nodes, kidney and colon. In patients with sporadic LAM, identification of the same mutations in renal AMLs and LAM cells led to the hypothesis that these two conditions are genetically interrelated. Although pathologically benign, LAM can cause severe complications including pneumothorax or chylothorax and may require surgical intervention and even lung transplantation. Two lesions believed to be malignant precursors of lung carcinoma, atypical adenomatous hyperplasia and multifocal micronodular pneuomocyte hyperplasia, have also been described in TSC patients.

Oral Cavity

Gingival fibromas and multiple enamel pits are the oral manifestations of TSC. Dental enamel pitting is seen in 90% of the TSC patients compared to 9% of the general population. Gingival

fibromas are usually located on the anterior gingival border, lips, dorsum of the tongue, palate, and other parts of the buccal mucosa and may be obscured by gingival hyperplasia.

Age	Lesion/Sign
Between 20th week of gestation and birth	Cardiac rhabdomyoma
Perinatal period	Subependymal nodule/tumor
	Multiple renal cysts
	Hydrops fetalis
	Seizures in utero
	Wolf-Parkinson-White syndrome
Newborn period	Fibrous forehead plaque
	Partial seizures with or without generalization
	Abdominal distention/uremia
Infancy	Infantile spasms/West syndrome
	Hypomelanotic macules
	Retinal hamartomas
	Regression of social-adaptive behaviour
	Delayed motor development
Early childhood	Seizures/Lennox-Gastaut syndrome
	Autism/atypical autism/mental handicap
	Facial angiofibromas
	Aberrant behavior/learning disability
Late childhood	Mental retardation/autism/intractable seizures
	Subependymal nodules/giant cell astrocytoma
	Status epilepticus
Adulthood	Late-onset seizures; status epilepticus
	Facial angiofibromas
	Renal AML causing hematuria or sudden bleed
	Pulmonary LAM
	Pneumothorax/chylothorax/respiratory failure/hypoxemia/ hypercarbia
	Ungual fibromas

Table 1: The relative age at which different types of TSC lesions and signs are most likely to be manifest clinically or by imaging methods [37]

Diagnostic Criteria of TSC

The criteria for the diagnosis of TSC are based on clinical and pathological examinations, and several imaging methods. Since the clinical spectrum of TSC is very diverse and variable, even within families, the clinical diagnostic criteria were divided in two groups: major and minor features (table 2).

A definite diagnosis of TSC can be made by the presence of either two major features or one major and two minor features (table 3). With this revision of the diagnostic criteria, no single feature of TSC is treated as pathognomonic.

Major Features
Facial angiofibromas or forehead plaques
Ungual or periungual fibromas
Hypomelanotic macules
Shagreen patch
Multiple retinal nodular hamartomas
Cortical tuber
Subependymal nodule
Subependymal giant cell astrocytoma
Cardiac rhabdomyoma
Lymphangiomyomatosis
Renal angiomyolipomas
Minor Features
Pits in dental enamel
Hamartomatous rectal polyps
Bone cysts
Cerebral white matter radial migration lines
Gingival fibromas
Nonrenal hamartomas
Retinal achromic patch
“Confetti” skin lesions
Multiple renal cysts

Table 2: Clinical features of TSC [38]

Definitive TSC
2 major features, or 1 major and 2 minor features
Probable TSC
1 major and 1 minor features
Possible TSC
Either 1 major feature, or 2 or more minor features

Table 3: Diagnostic classification of TSC [38]

In the majority of cases, these diagnostic criteria will be sufficient to make a definite diagnosis of TSC. In some relatives of the index case however, the phenotypic expression of TSC might be minimal (Chapters 2.d and 2.e). Furthermore in some families TSC might have very mild expression (Chapter 2.d). Somatic mosaicism might, in addition, be responsible for a milder phenotype in some cases. The highly variable phenotypic expression of TSC raises the possibility that a proportion of TSC patients may be missed using these strict diagnostic criteria and the actual incidence of the disorder may be higher.

Mutation analysis might shed light on cases where the clinical picture is not completely clear (Chapters 2.a, 2.d and 2.e) and functional assays might aid in differentiating polymorphisms and pathologic mutations in more complicated situations (Chapter 2.e).

1.c. Genetics of TSC

The TSC1 and TSC2 genes

Until the end of 1980s, very little progress was made toward discovering the molecular basis of TSC. For many years, researchers tried to determine the biochemical defect in patients, to uncover the missing or malfunctioning protein with no success. Many genes have been cloned using their protein sequences to design probes for isolating cDNAs from libraries (e.g. the gene encoding α glucosidase, the lysosomal enzyme that was known to be deficient in Glycogenosis type II). Unfortunately, in many genetic diseases, the malfunctioning or missing protein is not known before the identification of the causative gene(s), as was the case for TSC. To circumvent this problem, new techniques were developed based on the physical location of genes in the genome, which is called positional cloning.

In positional cloning, the rough location of a disease gene on a chromosome, *locus*, must first be determined. Sometimes, cytogenetic studies reveal a chromosomal translocation or deletion, giving a clue as to where the disease-causing gene can be located. When there is no cytogenetic starting point, DNA from multiple affected and unaffected individuals from multi-generation families are tested for polymorphisms with a known localization for linkage analysis. Polymorphisms linked to the disease causing gene will be passed from one generation to the next in such a way that polymorphisms shared by all the affected members in a family and not shared by the unaffected individuals will point to the location of the disease causing gene.

Fryer et al. performed such a linkage analysis in 19 multigenerational TSC families establishing the linkage between the ABO blood group gene on chromosome 9q34 and a TSC causing gene [39]. However, there were families that did not show linkage to the same locus. Furthermore, there was some evidence for a TSC gene on chromosome 11 [40, 41]. In 1989 and 1990, a study of several families indicated locus heterogeneity as the cause of inconsistency [42-45]. In 1991, multiple investigators formed a collaboration to study five large multigenerational families that were not linked to 9q34. A genome wide search revealed linkage between a TSC gene and a polymorphic marker near the *PKD1* gene on chromosome 16 [46]. The loci were named *TSC1* (9q34) and *TSC2* (16p13.3).

In 1993, one year after the establishment of linkage between the *TSC2* and *PKD1* genes, an unbalanced translocation in a family with TSC and ADPKD, and a few large deletions involving the tip of the short arm of chromosome 16 in non-TSC patients accelerated the cloning of the *TSC2* gene and the adjacent *PKD1* gene [47].

The hunt for the *TSC1* gene, on the other hand, was a laborsome process and proved difficult due to lack of large genomic rearrangements and conflicting positional information. It took researchers some 10 years to identify the gene: development of contigs of the candidate region, verification of positional information, systematic gene identification throughout the entire region, large-scale genomic sequencing of the contig and finally comprehensive mutational analysis of the candidate genes led to the identification of the *TSC1* in 1997 [48].

The 55 kb *TSC1* gene has 23 exons, of which 21 encode a 130 kDa protein named hamartin through an 8.6 kb mRNA. The 41 coding exons of the *TSC2* gene encode a 200 kDa protein named tuberin through a 5.4kb mRNA.

Inheritance of TSC

TSC is an autosomal dominant disorder, which means an affected individual has a 50% chance of having an affected offspring. However, in most cases neither parent has the disorder nor the faulty gene, and the faulty gene is first detected in the affected individual. Such cases are either caused by a *de novo* mutation that happens early in the embryonic development or through a *de novo* mutation resulting in gonadal mosaicism in one of the parents (figure 1). Gonadal mosaicism is the result of an early embryonal mutation in the parent that occurs either in a germ cell predecessor that continues to divide or before the germ cell commitment resulting in a combination with somatic mosaicism (also called gonosomal mosaicism). Since very few cells are destined to form the embryo early in development, a mutation occurring before the blastocyst stage may appear as a complete mutant. Mutations occurring at a later stage of embryogenesis will appear as somatic mosaics that may or may not be accompanied by gonadal mosaicism. Individuals with a mutation in a portion of their reproductive cells, who have no signs or symptoms of TSC, have an increased risk of having an affected offspring compared to the risk of the population, and mosaicism, both somatic and gonadal, has to be taken into account in risk assessment of TSC patients and families.

Another factor that needs to be addressed in the risk assessment of TSC patients and families is the penetrance. Penetrance is defined as the probability that an individual who has the pathogenic genotype will have the clinical manifestations of the disease [49]. TSC is considered to have a near complete penetrance [50]. However, the highly variable and age-dependent phenotypic expression of the disease make it difficult to determine the exact penetrance of TSC.

Approximately two thirds of the TSC cases are sporadic and this high proportion of *de novo* mutations reflects high mutation rates in the *TSC* genes [51], mutation rates comparable to those of the genes involved in Duchenne muscular dystrophy [52] and neurofibromatosis [53].

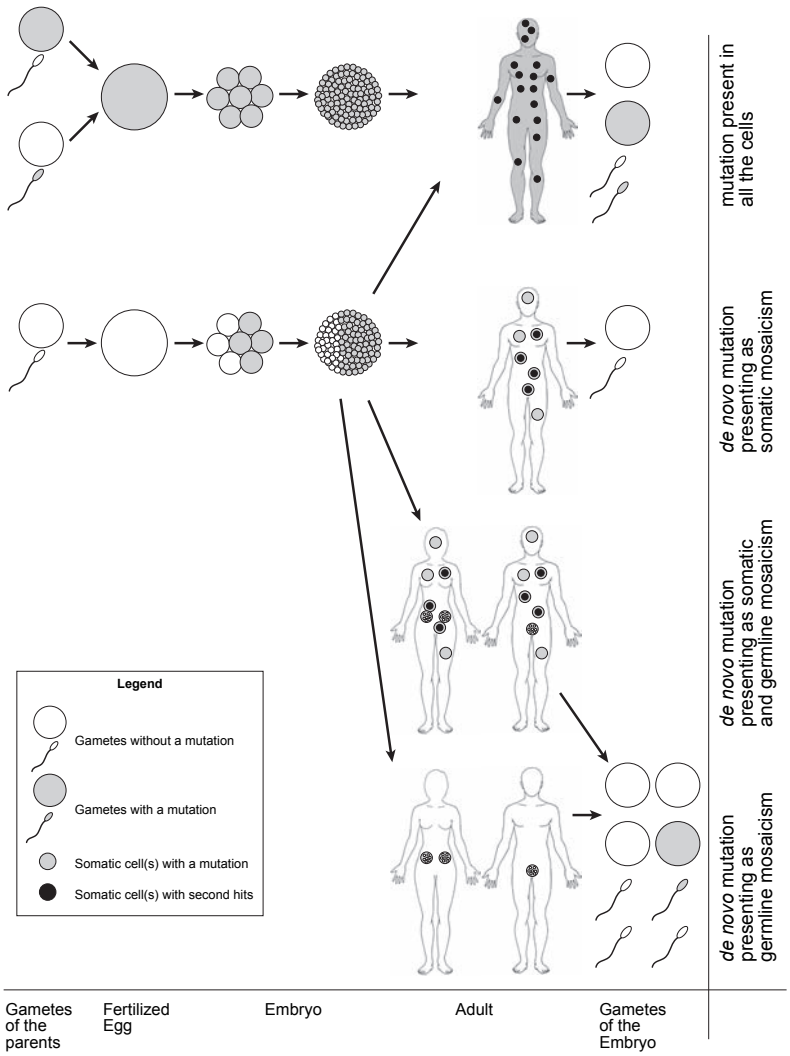


Figure 1: Inheritance of TSC. In the upper part of the figure, a mutation inherited from one of the parents is passed on to the offspring. In this case, all the cells of the offspring carry the germline mutation. In the lower part of the figure, a mutation arising during embryonal development is depicted. In this case, depending on how early the mutation arises, the mutation can affect virtually all the cells or result in somatic and/or gonadal mosaicism.

1.d. Mutation Analysis and Genotype-Phenotype Correlations in TSC

After the identification of the *TSC1* and *TSC2* genes, there has been a tremendous effort to identify disease causing mutations in both genes yielding a broad spectrum of mutations [47, 48, 54-60]. Interestingly, large rearrangements, missense mutations and in-frame deletions in the *TSC1* gene have rarely been reported (figure 2). Approximately 20% of the patients have a mutation in the *TSC1* gene and 80% in the *TSC2* gene. In *de novo* cases, *TSC1* mutations are

found two to ten times less often than *TSC2* mutations [57-60]. In contrast, in multi-generation families segregating TSC, approximately half show linkage to *TSC1* and half to *TSC2* [61]. A summary of the major mutation identification efforts is given in table 4 and types and frequencies of mutations identified in the reports, in which both *TSC1* and *TSC2* mutation analyses were performed, are shown in figure 2.

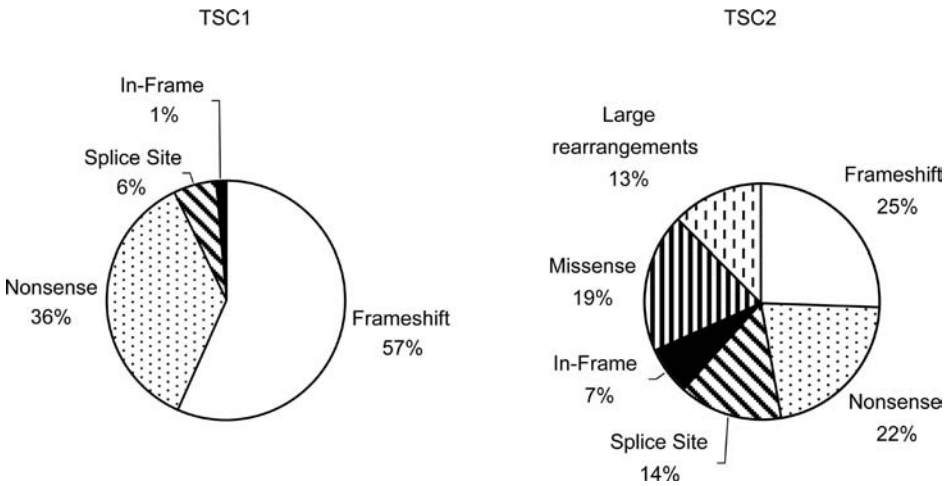


Figure 2: Types and frequencies of mutations identified in the reports in which both *TSC1* and *TSC2* mutation analyses were performed [57-60].

Pathogenic mutations can be identified in more than 80% of the cases using comprehensive mutation analysis. Different techniques have been used by different laboratories around the globe, including the protein truncation test (PTT), denaturing high-performance liquid chromatography (DHPLC), denaturing gradient gel electrophoresis (DGGE), single strand conformational polymorphism (SSCP), direct sequencing, fluorescent in situ hybridization (FISH) and Southern blot analysis. A combination of different techniques to detect small nucleotide changes as well as large genomic rearrangements is necessary to achieve high mutation detection rates.

Truncating nucleotide changes along both the *TSC1* and *TSC2* genes are accepted as pathogenic mutations. Non-truncating nucleotide changes on the other hand are not always pathogenic. Such mutations are predominantly found along the *TSC2* gene and it has proven difficult to establish their pathogenicity. Since approximately two thirds of the cases are caused by *de novo* mutations, the absence of a non-truncating nucleotide change in the unaffected parents of a TSC patient is a strong indication of pathogenicity. However, the presence of a non-truncating nucleotide change in one of the parents may or may not be an indication of an innocent polymorphism.

Somatic mosaicism and phenotypic heterogeneity within families might make it difficult to diagnose TSC in very mildly affected parents in some cases.

			TSC1		
First Author	**Year**	**Journal**	**Familial**	***de novo***	**Total**
Wilson, P.J.	1996	Hum Mol Genet	-	-	-
Au, K.	1997	Hum Mut	-	-	-
van Bakel, I.	1997	Hum Mol Genet	-	-	-
Jones, A.C.	1997	Hum Mol Genet	9	13	22
Au, K.	1998	Am J Hum Genet	-	-	-
Kwiatkowska, J.	1998	Ann Hum Genet	8	13	21
Beauchamp, R.L.	1998	Hum Mut	-	-	-
Gilbert, J.R.	1998	Neurogenetics	-	-	-
Jones, A.C.	1999	Am J Hum Genet	9	13	22
Mayer, K.	1999	Hum Mut	4	5	9
Benit, P.	1999	Hum Mut	3	2	5
van Slegtenhorst, M.	1999	J Med Genet	13	16	29
Niida, Y.	1999	Hum Mut	11	5	16
Dabora, S.L.	2001	Am J Hum Genet	5	22	28
Sancak, O.*	2005	Eur J Hum Genet	20	22	82

Table 4: Largest mutation analysis reports published to date. (SSCP: Single strandconformational polymorphism, SB: Southern blotting, PTT: Protein truncation test, PFGE: Pulsed-field gel electrophoresis, HA: Heteroduplex analysis, DHPLC: Denaturing high-performance liquid chromatography, LR-PCR: Long-range PCR, Q-PCR: Quantitative PCR, DGGE: Denaturing gradient gel electrophoresis, FISH: Fluorescence in situ hybridization, DS: Direct sequencing, *: Chapter 2.a. of this thesis)

TSC2				
Familial	*de novo*	Total	Total Number of patients	Methods Used
2	7	9	30	SSCP
2	-	2	88	SSCP, SB
1	4	5	18	PTT
2	47	49	171	PFGE, SB, SSCP, HA
3	18	21	90	SSCP
-	-	-	161	SSCP, HA, SB
10	11	21	40	SSCP
5	11	16	42	SSCP, PTT
9	88	98	150	SSCP, HA, SB, PFGE
1	18	19	48	PTT
-	-	-	15	SSCP, PTT
-	-	-	225	SSCP, SB
16	42	58	126	SSCP
22	129	158	224	DHPLC, LR-PCR, Q-PCR
43	115	280	490	SSCP, DGGE, SB, FISH, DS

Determining the pathogenicity of non-truncating nucleotide changes identified in a TSC patient, where the parents are not available or where one of the parents carries the same nucleotide change, is not possible based on genetic data and will require the use of more sophisticated measures to investigate if the change/loss/addition of the amino acid(s) leads to protein dysfunction.

When a pathogenic mutation is identified in an index patient, testing the other family members becomes feasible and may reveal very mildly affected, seemingly healthy relatives unaware of their condition. Identification of a pathogenic mutation will also help make a definite diagnosis in cases of doubt. A correct diagnosis is vital for TSC patients, mildly or severely affected alike, for appropriate medical care and follow-up, genetic counseling and the option of prenatal testing.

Many studies have focused on the phenotypic expression of the *TSC1* and *TSC2* gene mutations. Although initially the broad phenotypic and genotypic spectra of TSC made it difficult to establish an association, studies of larger cohorts indicated differences between TSC caused by *TSC1* and *TSC2* mutations. Jones et al. reported more frequent intellectual disability in sporadic patients with *TSC2* mutations compared to those with *TSC1* mutations, and provided the first reliable data on genotype-phenotype correlation in TSC [57]. A few years later, Dabora et al., published a more detailed report on a larger cohort of TSC patients [58]. In this study, clinical manifestations were found to be more frequent and/or more severe in patients with *de novo TSC2* mutations compared to those with *de novo TSC1* mutations. Seizures were significantly more common, average tuber and SEN counts were higher and on average, mental retardation was more severe in patients with *TSC2* mutations. In addition, the groups of patients with *TSC1* and *TSC2* mutations differed in the severity of dermatological manifestations and the frequency of renal AMLs, retinal hamartomas and LAM. The authors also showed that although the patients with *de novo TSC1* mutations had similar ages (range, average and median) to those with *de novo TSC2* mutations, patients with *de novo TSC1* mutations who had renal cysts, ungual fibromas, or facial angiofibromas were older compared to patients with *de novo TSC2* mutations with the same conditions. This was also the first extensive report to highlight possible correlations between different clinical features, including mental retardation – seizures, tuber count – mental retardation and renal AMLs – mental retardation.

The conflicting results of smaller and larger cohorts, and the lack of reliable and detailed genotype-phenotype correlations were the incentive to study a large independent TSC cohort (Chapter 2.a).

1.e. Functions of the *TSC1* and *TSC2* gene products

Hamartin and tuberin have been shown to associate physically and form a functional complex [62, 63]. Hamartin, the protein product of the *TSC1* gene, has a transmembrane domain (amino acids 127-144) and a large coiled-coil domain (amino acids 730-996) and has no homology to any other known protein (figure 3). Tuberin, the protein product of the *TSC2* gene, has a leucine zipper (amino acids 81-98), two coiled-coil domains (amino acids 346-371 and 1008-1021) and a GTPase activating protein (GAP) homologue domain (amino acids 1517-1674) (figure 4). Both

proteins act as tumor suppressors, since loss of heterozygosity (LOH) has been shown in many -but not all- TSC associated lesions. Tumor development results from the loss of the wild-type allele through a somatic "second hit" mutation according to Knudson's model. Many proteins have been shown to interact with either one of the proteins or with the hamartin-tuberin complex (table 5).

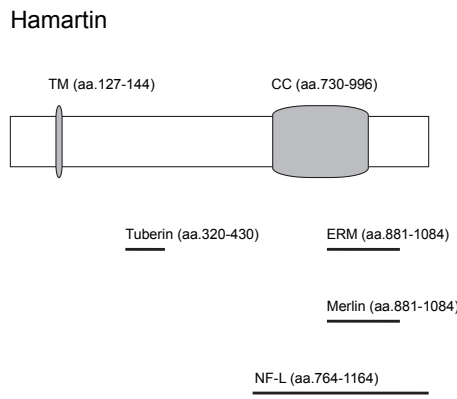


Figure 3: Hamartin: Structure and protein interactions. Hamartin, the product of *TSC1* gene, consists of 1164 amino acids. It has a potential transmembrane domain (TM, amino acids 127-144) and a coiled-coil domain (CC, amino acids 730-996). Proteins known to interact with hamartin are depicted below the figure. Figure is drawn to proportion.

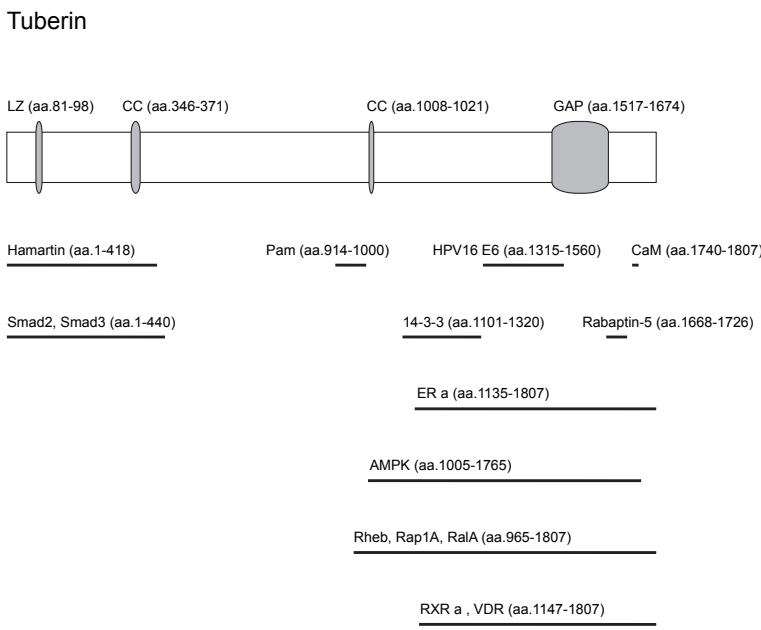


Figure 4: Tuberin: Structure and protein interactions. Tuberin, the product of *TSC2* gene, consists of 1807 amino acids. It has a leucine zipper region (LZ, amino acids 81-98), two coiled-coil domains (CC, amino acids 346-371 and 1008-1021), and a GTPase activating protein (GAP) homology region (GAP, amino acids 1517-1674). Proteins known to interact with tuberin are depicted below the figure. Figure is drawn to proportion.

Recent research linked the *TSC* gene products to the insulin/mTOR signaling pathway, one of the central pathways regulating cell size in response to growth factors, cellular energy and nutrient levels (figure 5) [64-66]. The activation of the pathway through growth-promoting stimuli results in increased translation of mRNAs that are specifically regulated via the translation initiation

factor eIF4E in addition to phosphorylation and activation of the 40S ribosomal protein (S6) also facilitating the translation of specific mRNAs. The key players of this cascade involve PI3K, the Akt kinase (PKB), and the mammalian target of rapamycin (mTOR).

The hamartin-tuberin complex is inhibited by the phosphorylation of tuberin by Akt [67] and is activated by the phosphorylation of tuberin by AMP-activated protein kinase (AMPK) [68] (see also Chapter 2 appendix). The complex, when active, acts as a GAP against Ras homolog enriched in brain (Rheb) and Rheb [66, 69, 70], when loaded with GTP, turns on mTOR kinase activity. Activated mTOR then phosphorylates p70S6 kinase (S6K), which in turn phosphorylates and activates S6, and 4E-binding protein (4EBP-1) which dissociates and thereby activates eIF4E (figure 5).

		binds to		suggested
Protein		**hamartin**	**tuberin**	**binding site(s)**
14-3-3 isoforms		-	+	Akt-phosphorylated tuberin MK2-phosphorylated tuberin TSC2 S1210
Akt	Protein kinase B	-	+	?
AMPK	5'-AMP-activated protein kinase	-	+	tuberin amino acids 1005-1765
CaM	Calmodulin		+	tuberin amino acids 1740-1758
Cdk1	Cyclin-dependent kinase 1	complex		?
Cyclin B1		complex		?
Cyclin A			+	?
ER a	Estrogen receptor a		+	tuberin amino acids 1135-1807
ERM	Ezrin, Radixin, Moesin	+		hamartin amino acids 881-1084
HPV16 E6	Huma papilloma virus E6 protein		+	tuberin amino acids 1315-1560
Merlin	Moesin-ezrin-radixin-like protein	+		hamartin amino acids 881-1084
mTOR	Mammalian target of rapamycin	complex		?
NF-L	Neurofilament light chain	+	-	hamartin amino acids 674-1164
Pam	Protein associated with Myc	-	+	tuberin amino acids 914-1000
Rabaptin-5	Rab GTPase binding effector protein 1		+	tuberin amino acids 1668-1726
Rheb, Rap1A, RalA	small GTPase proteins		+	tuberin amino acids 965-1807
RXR a, VDR	Retinoic acid receptor a, Vitamin D3 receptor		+	tuberin amino acids 1147-1807
Smad2, Smad3	Homologs of Mad2 and Mad3		+	tuberin amino acids 1-440

Table 5: Proteins shown/hypothesized to be interacting with tuberin and/or hamartin

The proteins involved in other hamartomatous syndromes have been mapped to the very same pathway[71]. LKB1, the tumor suppressor gene responsible for Peutz-Jeghers syndrome, phosphorylates and activates AMPK [72], which in turn phosphorylates and activates tuberin. On the other hand, PTEN, another tumor suppressor gene involved in several different hamartomatous syndromes, is a lipid phosphatase that negatively regulates the cell survival through the PI3K/Akt signaling [73].

Besides the role of hamartin-tuberin complex in cell size control, the complex also seems to be involved in cell cycle control. Cells lacking either protein have shortened G1 phase, and antisense inhibition of *TSC2* expression induces quiescent fibroblasts to enter cell cycle[74-77]. In addition, over-expression of *TSC1* or *TSC2* negatively regulates cell cycle progression. The complex has been shown to negatively regulate cdk2 activity and, when inactivated, decrease the stability of p27. Hamartin and tuberin have been postulated to interact with many proteins involved in cell cycle regulation involving cdk1, cyclin B, cyclin A, and p27. The hamartin-tuberin complex seems to be centrally located in different signaling pathways to help synchronize cell size control and cell cycle regulation.

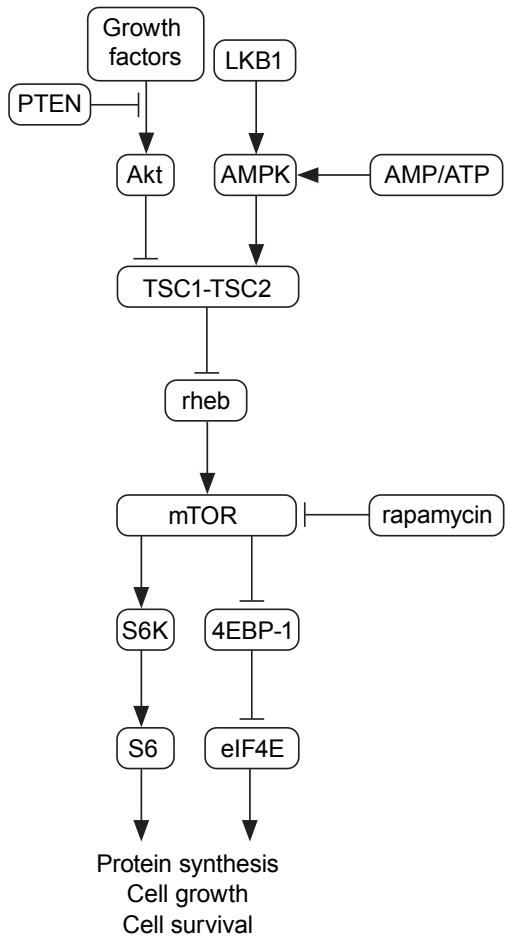


Figure 5: mTOR signaling pathway. Hamartin (TSC1: the product of *TSC1* gene) and tuberin (TSC2: the product of *TSC2* gene) form a complex that is inhibited by Akt and activated by AMPK. The hamartin-tuberin complex functions as a GTPase activating protein (GAP) complex towards rheb, and rheb, when loaded with GTP, activates mTOR to facilitate protein synthesis.

1.f. Scope of this Thesis

The TSC research group at the Erasmus Medical Center, formed originally to identify the responsible genes for this disorder, has become one of the major referral sites in the world for mutation analysis of the *TSC1* and *TSC2* genes. First aim of this thesis was to develop and apply a comprehensive mutation analysis strategy to analyse patients' DNA that has been referred to our center for over a decade and to perform a systematic study of genotype-phenotype correlation in the largest patient cohort known to date.

Mutation analysis in a disorder with high spontaneous mutation rate and no mutation hotspots, as is the case for TSC, will result in the identification of numerous different nucleotide changes. Characterization of such changes and determining whether such changes are pathogenic can be difficult, or impossible at times, based on genetic data only. The recent advances in our understanding of the functions of the tuberin-hamartin complex in the signaling cascade enabled biochemical assays to analyze non-truncating mutations. Second aim of this thesis was to develop and apply reliable functional assays for the characterization of such mutations in the *TSC2* gene and to determine if it is feasible to use such assays in a diagnostic manner.

Third aim of this thesis was to gain a deeper understanding of the functions of the tuberin-hamartin complex, how the complex is activated and what the consequences of this activation are.

The research presented in this thesis is intended to help clinicians, researchers, and individuals and families with TSC, by providing deeper insights into the mutational spectrum of the *TSC1* and *TSC2* genes, phenotypic variability, disease penetrance, and functions of the hamartin-tuberin complex.

References

1. Rayer, P.F.O., *Traité theorique et pratique des maladies de la peau.* 2nd ed, ed. Bailliére. 1835, Paris.
2. Balzer, F. and P. Ménétrier, *Étude sur un cas d'adénomes sébacés de la face et du cuir chevelu.* Arch Physiol Norm Pathol (série III), 1885. **6**: p. 564-576.
3. von Recklinghausen, F., *Ein Herz von einem Neugeborene welches mehrere theils nach aussen, theils nach den Höhlen prominirende Tumoren (Myomen) trug.* Monatschr Geburtsheilkd, 1862. **20**: p. 1-2.
4. Bourneville, D.M., *Sclérose tubéreuse des circonvolutions cérébrales: idiotie et épilepsie hémiplégique.* Arch Neurol (Paris), 1880. **1**: p. 81-91.
5. Bourneville, D.M. and E. Brissaud, *Encéphalite ou sclérose tubéruese des circonvolutions cérébrales.* Arch Neurol (Paris), 1881. **1**: p. 390-410.
6. Vogt, H., *Zur pathologie und pathologischen Anatomie der verschiedenen Idiotieform.* Monatsschr Psychiatr Neurol, 1908. **24**: p. 106-150.
7. Bielschowsky, M., *Übertuberose Sklerose und ihre Beziehungen zur Recklinghausenschen Krankheit.* Z Ges Neurol Psychiatr, 1914. **26**: p. 133-155.

8. Pringle, J.J., *A case of congenital adenoma sebaceum.* Br J Dermatol, 1890. **2**: p. 1-14.
9. Campbell, A.W., *Cerebral sclerosis.* Brain, 1905. **28**: p. 382-396.
10. Pellizzi, G.B., *Contributo allo studio del idiozia.* Riv Sperim Freniat, 1901. **27**: p. 265-9.
11. Perusini, G., *Über einen Fall von Sclerosis tuberosa hypertrophica.* Monatsschr Psychiatr Neurol, 1905. **17**: p. 69-255.
12. Kirpicznik, J., *Ein Fall von Tuberoser Sklerose und gleichzeitigen multiplen Nierengeschwülsten.* Virchow Arch Pathol Anat, 1910. **202**(3): p. 358.
13. Berg, H., *Vererbung der Tuberösen Sklerose durch zweigzu drei Generationen.* Z Ges Neurol Psychiatr, 1913. **19**: p. 528-539.
14. Schuster, P., *Beiträge zur Klinik der tuberösen Sklerose des Gehirns.* Dtsch Z Nervenheilkd, 1914. **50**: p. 96-133.
15. van der Hoeve, J., *Eye symptoms in tuberous sclerosis of the brain.* Trans Ophthalmol Soc U K, 1920. **20**: p. 329-334.
16. Marcus, H., *Svenska Lak Sallsk Forh. 1924; 1412, cited by Dickerson WW. Characteristic roentgenographic changes associated with tuberous sclerosis.* Arch Neurol Psychiatry, 1945. **53**: p. 199-204.
17. Dalsgaard-Nielsen, T., *Tuberous sclerosis with unusual roentgen picture.* Nord Med, 1935. **10**: p. 1541-1548.
18. Berkowitz, N.J. and L.G. Rigler, *Tuberous sclerosis diagnosed with cerebral pneumography.* Arch Neurol Psychiatry, 1934. **35**: p. 833-838.
19. Critchley, M. and C.J.C. Earl, *Tuberose sclerosis and allied conditions.* Brain, 1932. **55**: p. 311-346.
20. Moolten, S.E., *Hamartial nature of the tuberous sclerosis complex and its bearing on the tumor problem: report of one case with tumor anomaly of the kidney and adenoma sebaceum.* Arch Intern Med, 1942. **69**: p. 589-623.
21. Lagos, J.C. and M.R. Gomez, *Tuberous sclerosis: reappraisal of a clinical entity.* Mayo Clin Proc, 1967. **42**(1): p. 26-49.
22. Gomez, M.R., *Criteria for diagnosis*, in *Tuberous Sclerosis*, M.R. Gomez, Editor. 1988, Raven Press: New York. p. 9-19.
23. Donegani, G., F.R. Gratarolla, and E. Wildi, *Tuberous sclerosis*, in *Handbook of Clinical Neurology*, P.J. Vinken and G.W. Bruyn, Editors. 1972, North Holland: Amsterdam. p. 340-389.
24. Osborne, J.P., A. Fryer, and D. Webb, *Epidemiology of tuberous sclerosis.* Ann N Y Acad Sci, 1991. **615**: p. 125-7.
25. Gomez, M.R., *Tuberous Sclerosis.* 1st ed, ed. M.R. Gomez. 1979, New York: Raven Press.
26. Roach, E.S., et al., *Diagnostic criteria: tuberous sclerosis complex. Report of the Diagnostic Criteria Committee of the National Tuberous Sclerosis Association.* J Child Neurol, 1992. **7**(2): p. 221-4.
27. Neumann, H.P. and R.S. Kandt, *[The clinical picture and genetics of tuberous sclerosis]*
Klinik und Genetik der tuberosen Sklerose. Dtsch Med Wochenschr, 1993. **118**(43): p. 1577-83.

28. Roach, E.S., M.R. Gomez, and H. Northrup, *Tuberous sclerosis complex consensus conference: revised clinical diagnostic criteria.* J Child Neurol, 1998. **13**(12): p. 624-8.
29. Humphrey, A., et al., *Monozygotic twins with tuberous sclerosis discordant for the severity of developmental deficits.* Neurology, 2004. **62**(5): p. 795-8.
30. Martin, N., et al., *Discordant clinical manifestations in monozygotic twins with the identical mutation in the TSC2 gene.* Clin Genet, 2003. **63**(5): p. 427-30.
31. Smalley, S.L., F. Burger, and M. Smith, *Phenotypic variation of tuberous sclerosis in a single extended kindred.* J Med Genet, 1994. **31**(10): p. 761-5.
32. Sampson, J.R., et al., *Renal cystic disease in tuberous sclerosis: role of the polycystic kidney disease 1 gene.* Am J Hum Genet, 1997. **61**(4): p. 843-51.
33. Isaacs, H., Jr., *Fetal and neonatal cardiac tumors.* Pediatr Cardiol, 2004. **25**(3): p. 252-73.
34. Costello, L.C., T.E. Hartman, and J.H. Ryu, *High frequency of pulmonary lymphangioleiomyomatosis in women with tuberous sclerosis complex.* Mayo Clin Proc, 2000. **75**(6): p. 591-4.
35. Matsumoto, Y., et al., *Markers of cell proliferation and expression of melanosomal antigen in lymphangioleiomyomatosis.* Am J Respir Cell Mol Biol, 1999. **21**(3): p. 327-36.
36. McCormack, F., et al., *Pulmonary cysts consistent with lymphangioleiomyomatosis are common in women with tuberous sclerosis: genetic and radiographic analysis.* Chest, 2002. **121**(3 Suppl): p. 61S.
37. Gomez, M., J. Sampson, and V. Whittemore, *The Tuberous Sclerosis Complex*. 1999, Oxford, England: Oxford University Press.
38. Roach, E.S., et al., *Tuberous Sclerosis Consensus Conference: recommendations for diagnostic evaluation. National Tuberous Sclerosis Association.* J Child Neurol, 1999. **14**(6): p. 401-7.
39. Fryer, A.E., et al., *Evidence that the gene for tuberous sclerosis is on chromosome 9.* Lancet, 1987. **1**(8534): p. 659-61.
40. Smith, M., et al., *Mapping of a gene determining tuberous sclerosis to human chromosome 11q14-11q23.* Genomics, 1990. **6**(1): p. 105-14.
41. Smith, M., et al., *Genetic heterogeneity in tuberous sclerosis. Map position of the TSC2 locus on chromosome 11q and future prospects.* Ann N Y Acad Sci, 1991. **615**: p. 274-83.
42. Janssen, L.A., et al., *Genetic heterogeneity in tuberous sclerosis.* Genomics, 1990. **8**(2): p. 237-42.
43. Northrup, H., et al., *Evidence for genetic heterogeneity in tuberous sclerosis: one locus on chromosome 9 and at least one locus elsewhere.* Am J Hum Genet, 1992. **51**(4): p. 709-20.
44. Sampson, J.R., et al., *Evidence for genetic heterogeneity in tuberous sclerosis.* J Med Genet, 1989. **26**(8): p. 511-6.
45. Haines, J.L., et al., *Localization of one gene for tuberous sclerosis within 9q32-9q34, and further evidence for heterogeneity.* Am J Hum Genet, 1991. **49**(4): p. 764-72.

46. Kandt, R.S., et al., *Linkage of an important gene locus for tuberous sclerosis to a chromosome 16 marker for polycystic kidney disease.* Nat Genet, 1992. **2**(1): p. 37-41.
47. *Identification and characterization of the tuberous sclerosis gene on chromosome 16. The European Chromosome 16 Tuberous Sclerosis Consortium.* Cell, 1993. **75**(7): p. 1305-15.
48. van Slegtenhorst, M., et al., *Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34.* Science, 1997. **277**(5327): p. 805-8.
49. Strachan, T. and A.P. Read, *Human Molecular Genetics*. Third Edition ed. 2004, Oxon, UK: Garland Publishing.
50. Kwiatkowski, D.J. and M.P. Short, *Tuberous sclerosis.* Arch Dermatol, 1994. **130**(3): p. 348-54.
51. Sampson, J.R., et al., *Genetic aspects of tuberous sclerosis in the west of Scotland.* J Med Genet, 1989. **26**(1): p. 28-31.
52. van Essen, A.J., et al., *Birth and population prevalence of Duchenne muscular dystrophy in The Netherlands.* Hum Genet, 1992. **88**(3): p. 258-66.
53. Friedman, J.M., *Epidemiology of neurofibromatosis type 1.* Am J Med Genet, 1999. **89**(1): p. 1-6.
54. van Bakel, I., et al., *Mutations in the TSC2 gene: analysis of the complete coding sequence using the protein truncation test (PTT).* Hum Mol Genet, 1997. **6**(9): p. 1409-14.
55. van Slegtenhorst, M., et al., *Mutational spectrum of the TSC1 gene in a cohort of 225 tuberous sclerosis complex patients: no evidence for genotype-phenotype correlation.* J Med Genet, 1999. **36**(4): p. 285-9.
56. Au, K.S., et al., *Germ-line mutational analysis of the TSC2 gene in 90 tuberous-sclerosis patients.* Am J Hum Genet, 1998. **62**(2): p. 286-94.
57. Jones, A.C., et al., *Comprehensive mutation analysis of TSC1 and TSC2-and phenotypic correlations in 150 families with tuberous sclerosis.* Am J Hum Genet, 1999. **64**(5): p. 1305-15.
58. Dabora, S.L., et al., *Mutational analysis in a cohort of 224 tuberous sclerosis patients indicates increased severity of TSC2, compared with TSC1, disease in multiple organs.* Am J Hum Genet, 2001. **68**(1): p. 64-80.
59. Niida, Y., et al., *Analysis of both TSC1 and TSC2 for germline mutations in 126 unrelated patients with tuberous sclerosis.* Hum Mutat, 1999. **14**(5): p. 412-22.
60. Langkau, N., et al., *TSC1 and TSC2 mutations in tuberous sclerosis, the associated phenotypes and a model to explain observed TSC1/ TSC2 frequency ratios.* Eur J Pediatr, 2002. **161**(7): p. 393-402.
61. Cheadle, J.P., et al., *Molecular genetic advances in tuberous sclerosis.* Hum Genet, 2000. **107**(2): p. 97-114.
62. van Slegtenhorst, M., et al., *Interaction between hamartin and tuberin, the TSC1 and TSC2 gene products.* Hum Mol Genet, 1998. **7**(6): p. 1053-7.
63. Nellist, M., et al., *Characterization of the cytosolic tuberin-hamartin complex. Tuberin is a cytosolic chaperone for hamartin.* J Biol Chem, 1999. **274**(50): p. 35647-52.

64. Potter, C.J., L.G. Pedraza, and T. Xu, *Akt regulates growth by directly phosphorylating Tsc2.* Nat Cell Biol, 2002. **4**(9): p. 658-65.
65. Gao, X. and D. Pan, *TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth.* Genes Dev, 2001. **15**(11): p. 1383-92.
66. Garami, A., et al., *Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2.* Mol Cell, 2003. **11**(6): p. 1457-66.
67. Inoki, K., et al., *TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling.* Nat Cell Biol, 2002. **4**(9): p. 648-57.
68. Inoki, K., T. Zhu, and K.L. Guan, *TSC2 mediates cellular energy response to control cell growth and survival.* Cell, 2003. **115**(5): p. 577-90.
69. Tee, A.R., et al., *Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb.* Curr Biol, 2003. **13**(15): p. 1259-68.
70. Zhang, Y., et al., *Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins.* Nat Cell Biol, 2003. **5**(6): p. 578-81.
71. Inoki, K., M.N. Corradetti, and K.L. Guan, *Dysregulation of the TSC-mTOR pathway in human disease.* Nat Genet, 2005. **37**(1): p. 19-24.
72. Shaw, R.J., et al., *The LKB1 tumor suppressor negatively regulates mTOR signaling.* Cancer Cell, 2004. **6**(1): p. 91-9.
73. Zhou, X.P., et al., *Germline inactivation of PTEN and dysregulation of the phosphoinositol-3-kinase/Akt pathway cause human Lhermitte-Duclos disease in adults.* Am J Hum Genet, 2003. **73**(5): p. 1191-8.
74. Tapon, N., et al., *The Drosophila tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation.* Cell, 2001. **105**(3): p. 345-55.
75. Uhlmann, E.J., et al., *Heterozygosity for the tuberous sclerosis complex (TSC) gene products results in increased astrocyte numbers and decreased p27-Kip1 expression in TSC2+/- cells.* Oncogene, 2002. **21**(25): p. 4050-9.
76. Soucek, T., et al., *Tuberous sclerosis causing mutants of the TSC2 gene product affect proliferation and p27 expression.* Oncogene, 2001. **20**(35): p. 4904-9.
77. Miloloza, A., et al., *Evidence for separable functions of tuberous sclerosis gene products in mammalian cell cycle regulation.* J Neuropathol Exp Neurol, 2002. **61**(2): p. 154-63.

2

PUBLICATIONS

MUTATIONAL ANALYSIS OF THE *TSC1* AND *TSC2* GENES IN A DIAGNOSTIC SETTING: GENOTYPE – PHENOTYPE CORRELATIONS AND COMPARISON OF DIAGNOSTIC DNA TECHNIQUES IN TUBEROUS SCLEROSIS COMPLEX

Ozgur Sancak[1], Mark Nellist[1], Miriam Goedbloed[1], Peter Elfferich[1], Cokkie Wouters[1], Anneke Maat-Kievit[1], Bernard Zonnenberg[3], Senno Verhoef[2], Dicky Halley[1] and Ans van den Ouweland*[1]

[1]Department of Clinical Genetics, Erasmus Medical Center, Rotterdam, The Netherlands;
[2]Family Cancer Clinic, Netherlands Cancer Institute, Amsterdam, The Netherlands;
[3]TSC Clinic, Department of Medical Oncology, University Medical Center Utrecht, Utrecht, The Netherlands

European Journal of Human Genetics (2005), 1–11

Received 27 October 2004; revised 4 February 2005; accepted 8 February 2005

European Journal of Human Genetics advance online publication, 30 March 2005; doi:10.1038/sj.ejhg.5201402

*Correspondence: Dr A van den Ouweland,
Department of Clinical Genetics,
Erasmus Medical Center,
Dr Molewaterplein 50,
3015 GE Rotterdam, The Netherlands.
Tel: +31 10 4087197;
Fax: +31 10 4089489;
E-mail: a.vandenouweland@erasmusmc.nl

ABSTRACT

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterised by the development of hamartomas in multiple organs and tissues. TSC is caused by mutations in either the *TSC1* or *TSC2* gene. We searched for mutations in both genes in a cohort of 490 patients diagnosed with or suspected of having TSC using a combination of denaturing gradient gel electrophoresis, single-strand conformational polymorphism, direct sequencing, fluorescent in situ hybridisation and Southern blotting. We identified pathogenic mutations in 362 patients, a mutation detection rate of 74%. Of these 362 patients, 276 had a definite clinical diagnosis of TSC and in these patients 235 mutations were identified, a mutation detection rate of 85%. The ratio of *TSC2*:*TSC1* mutations was 3.4:1. In our cohort, both *TSC1* mutations and mutations in familial *TSC2* cases were associated with phenotypes less severe than *de novo TSC2* mutations. Interestingly, consistent with other studies, the phenotypes of the patients in which no mutation was identified were, overall, less severe than those of patients with either a known *TSC1* or *TSC2* mutation.

Keywords: *TSC1* and *TSC2*; tuberous sclerosis complex; genotype–phenotype correlation

INTRODUCTION

Tuberous sclerosis complex (TSC, MIM #191,100), affecting between 1/6,000 and 1/10,000 individuals, is an autosomal dominant disorder characterised by seizures, mental retardation and the development of hamartomas in multiple organs and tissues[1]. In approximately two-thirds of cases neither parent has signs of TSC and the disease is caused by a *de novo* mutation, indicating a high rate of spontaneous mutation in the causative genes, *TSC1* and *TSC2*.

The *TSC1* gene consists of a 3.4-kb open reading frame encoded by 21 exons, whereas the 5.4-kb open reading frame of the *TSC2* gene is encoded by 41 exons. Loss of heterozygosity across the TSC1 and TSC2 loci in TSC-associated lesions indicates that *TSC1* and *TSC2* are tumor suppressor genes[2]. The TSC1 and TSC2 gene products, hamartin and tuberin, form a complex[3] that activates the GTPase activity of rheb, preventing the rheb-GTP-dependent stimulation of cell growth through mTOR[4].

A broad spectrum of mutations has been identified in both genes[5-10]. Interestingly, large rearrangements, missense mutations and in-frame deletions are very rare in the *TSC1* gene. The majority of TSC patients have a mutation in the *TSC2* gene. In *de novo* cases, mutations in the *TSC2* gene are found two to ten times more often than *TSC1* mutations[9-12]. In contrast, in multi-generation families segregating TSC, approximately half show linkage to TSC1 and half to TSC2[13]. It has been suggested that this may be because TSC patients with a *TSC1* mutation are less likely to be severely affected and therefore more likely to have offspring[12]. Although initially the broad phenotypic and genotypic spectra of TSC made it difficult to establish an association[7, 11], studies of larger cohorts indicated that patients with a *TSC1* mutation were less likely to be mentally retarded[9], while patients with a *TSC2* mutation had a higher number and/or severity of clinical features[10].

We have performed a comprehensive screen for mutations at the TSC loci and compared the phenotypes within a large unrelated TSC patient cohort including the previously described group of patients[7]. The majority of TSC patients in our cohort have a mutation in the *TSC2* gene, and, overall, *TSC2* mutations are associated with a more severe TSC phenotype. Our analysis confirms and extends the findings of previous studies.

MATERIALS AND METHODS

Patients

Samples from patients with a (putative) diagnosis of TSC were obtained via several specialists in the Netherlands and abroad. A standardised clinical evaluation form was sent to all the referring clinicians. In familial cases, only index patients were used for phenotypic comparisons.

Mutation analysis

DNA was extracted from peripheral blood cells using standard techniques. Mutation analysis was performed using a combination of single-strand conformational polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), direct sequencing, Southern blotting and fluorescence *in situ* hybridisation (FISH).

SSCP, Southern and FISH analyses were performed as described previously[14, 15]. For DGGE, primers were designed using the Ingeny DGGE Primer Design program (primer sequences available upon request), and pooled PCR products were run on urea/formamide denaturing gradient polyacrylamide gels at 60°C for 17 h (Ingeny phorU DGGE system). SSCP was performed on 50% of the patients and DGGE on 75% of the cohort. One exon in *TSC1* and five exons in *TSC2* were not suitable for DGGE and were sequenced. If no mutation was identified, Southern blotting and FISH analysis were undertaken. Direct sequencing of M13-tagged PCR products was carried out on an ABI3100 capillary sequencer using Big Dye Terminator v 3 chemistry (Applied Biosystems). A nucleotide change was classified as a polymorphism when this change was present in the patient together with a pathogenic mutation, when it was present in the DNA of the unaffected parent of the patient or when it had been classified as a polymorphism in the literature. A complete overview of our mutation analysis strategy is available upon request.

Total RNA was isolated from cultured patient fibroblasts. RT-PCR was performed according to standard procedures. Functional analysis of the effect of *TSC2* missense changes on the tuberin–hamartin complex was performed as described previously[16, 17].

Statistical analysis

Statistical comparisons were performed using the SPSS version 11.0 package for PC. Pearson χ^2 or Fisher's exact test was used for comparisons of categorical data and Mann–Whitney test was used for the comparison of median age at referral.

RESULTS

Patient characteristics

Mutation analysis of the *TSC1* and *TSC2* genes was performed on 490 patients with a (putative) diagnosis of TSC (Table 1). In 276 cases, sufficient clinical information was obtained to make a definite diagnosis of TSC according to the criteria defined by Roach et al[18]. In 15 cases there was insufficient data for a definite diagnosis and in 199 cases we did not receive any clinical data. The 291 patients with clinical information were between 0 and 60 years old at the time of referral, with a median age of 13.0.

	Total	Mutation identified	Unclas-sified variant	No mutation identified	TSC1 mutation	TSC2 mutation	*de novo*	familial
Patients with clinical information								
Def. TSC	276	235	14	27	53	182	92	50
Insuf. information	15	4	1	10	1	3	1	1
Total	291	239	15	37	54	185	93	51
No clinical information	199	123	14	62	28	95	44	12
Total	490	362	29	99	82	280	137	63

Table 1: Overview of the TSC patient cohort. Groups with a definite diagnosis of TSC (Def. TSC), insufficient clinical information for a definite diagnosis (Insuf. information) and without any clinical information (No clinical information) are shown. For each group, patients with mutations in the TSC1 and the TSC2 genes in addition to patients with nucleotide changes that could not yet be classified as pathogenic (unclassified variant) are depicted. The number of patients with de novo mutations and of patients of familial cases (based on molecular analysis) are also shown.

We examined the correlation between different clinical features in the 276 definitive TSC patients. The majority of the patients were mentally retarded (166/276; 60%), and 163 of these patients (98%) had seizures. In contrast, only 37 of the 63 patients without mental retardation had seizures (59%). This difference was significant ($P<0.001$). Seizures were present in 86% of the patients with (sub)-cortical tubers and in 56% of the patients free of tubers ($P=0.004$). The presence of (sub)cortical tubers also correlated with the presence of mental retardation. Of the patients with (sub)cortical tubers 82% had mental retardation, while only 33% of the patients without tubers were mentally retarded ($P=0.002$). Subependymal nodules were noted in 88% of the patients with (sub)cortical tubers and in 63% of the patients without tubers ($P=0.013$). In addition, there was a significant correlation between the incidence of renal angiomyolipomas and mental retardation in patients above the age of 6 years at referral. The patients with renal angiomyolipoma (82%) had more frequently mental retardation compared to the patients without renal angiomyolipoma (64%)($P=0.041$).

Comparison of the clinical features between male and female subjects showed no significant difference in the median age of the two groups ($P=0.467$) (Table 2). However, 75% of the clinical

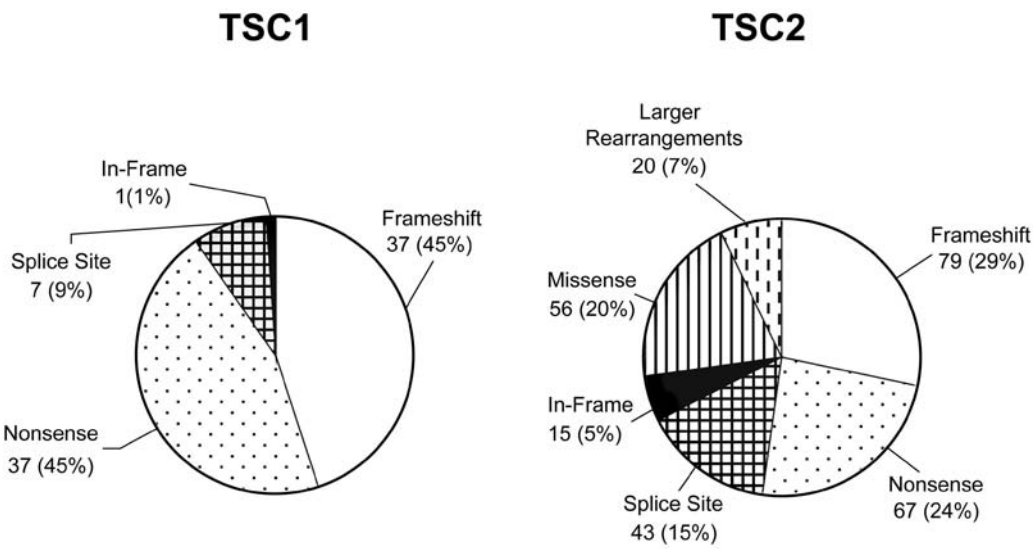
features analysed were more frequent in males. Mental retardation (P=0.012), facial angiofibromas (P=0.050), retinal phakomas (P=0.025), retinal depigmentations (P=0.005) and gingival fibromas (P=0.019) were noted significantly more frequently in males. Lymphangioleiomyomatosis (LAM) was only detected in females (four cases). However, due to the low numbers of patients examined for LAM (12 males and 21 females), this difference was not significant (P=0.146).

	Male		Female		
	P/N	%	P/N	%	p values
Mental retardation	89/108	83	63/93	68	0.012*
Seizures	110/121	91	90/104	87	0.204
Cortical tubers	56/63	89	56/62	90	0.512
SEN	84/93	90	73/82	89	0.485
SEGA	9/30	30	13/37	35	0.429
Renal AML	31/74	42	33/70	47	0.321
Renal cysts	22/67	33	12/62	19	0.062
Shagreen patch	30/56	54	30/61	49	0.386
Hypomelanotic macules	94/102	92	80/90	89	0.299
Forehead plaque	23/53	43	17/50	34	0.219
Facial angiofibromas	82/97	85	61/83	74	0.050*
Ungual fibroma	28/64	44	17/59	29	0.063
Retinal phakoma	26/69	38	11/55	20	0.025*
Retinal depigmentation	11/20	55	2/18	11	0.005*
Gingival fibroma	10/17	59	5/23	22	0.019*
Dental pits	20/32	63	19/37	51	0.246
Cardiac rhabdomyoma	23/58	40	20/53	38	0.495
LAM	0/19	0	4/21	19	0.146

Table 2: Comparison of the clinical features of TSC associated with gender. For each group, the first column shows the number of patients with the feature (P) and the total number of patients examined (N) and the second column shows the frequency of each clinical feature (%). * Indicates statistically significant values. Median age was 14.0 yrs for males and 13.0 yrs for females (p=0.467). (SEN; subependymal nodules, SEGA; subependymal giant cell astrocytoma, AML; angiomyolipoma, LAM; lymphangioleiomyomatosis).

Mutation analysis

We identified pathogenic mutations in 362 patients (74% of the total cohort), 82 mutations in the *TSC1* gene and 280 in the *TSC2* gene (23% vs 77%). The different types of *TSC1* and *TSC2* mutations are shown in Figure 1. In addition, we identified 20 different polymorphisms in the *TSC1* gene and 56 in the *TSC2* gene. In 29 cases (6% of the total), we detected nucleotide changes in the *TSC2* gene, but were unable to establish whether they were pathogenic. A complete overview of all the changes identified at both loci is available on request.



When possible, the parents of the patient were tested for the presence of a (putative) pathogenic mutation (Table 1). In 137 cases (22 *TSC1* and 115 *TSC2*), the change identified in the patient was not present in the DNA of either parent. We defined these cases as *de novo* TSC. In 63 cases (20 *TSC1* and 43 *TSC2*) the same pathogenic change was identified in the affected parent of the patient. In 10 cases, we could not rule out the possibility that a change identified in both the patient and another affected parent was nonpathogenic. In 181 cases the parents were not available for testing.

Four nucleotide changes in the *TSC2* gene showed a possible effect on RNA splicing using three different splice-site prediction programs (www.fruitfly.org/seq_tools/splice.html; www.cbs.dtu.dk/services/NetGene2 and www.genet.sickkids.on.ca/~ali/splicesitefinder.html). RTPCR analysis showed an incorrectly spliced TSC2 mRNA in three cases, confirming that the changes were pathogenic. In one case (IVS5+4A>G) we were unable to detect any abnormally spliced variant. However, further investigation revealed that the expression of the mutant allele was severely reduced. In several cases the effects of TSC2 missense mutations on tuberin function were investigated. Missense changes that inactivated tuberin in vitro were classified as pathogenic. In cases where we could not demonstrate that missense and intronic changes were *de novo* or had an effect on splicing or the function of the tuberin–hamartin complex, we designated the changes as ‘unclassified variants’.

Previously we described two cases of mosaicism at the TSC1 locus and four cases at the TSC2 locus in the parents of TSC patients[14]. We have identified two additional cases of mosaicism in the *TSC2* gene, one large *de novo* deletion in a patient (80% of the cells) and one nonsense mutation (3112C4T (R1032X)) in the father of a familial case of TSC.

In 94 patients where both SSCP and DGGE were performed and a mutation was identified, 87

mutations (93%) were identified by DGGE, and 79 mutations (84%) were identified by SSCP. In 71 (76%) cases the mutation was identified by both techniques. In the *TSC2* gene, seven changes were detected by SSCP but not by DGGE, while seven changes in the *TSC1* gene and eight changes in the *TSC2* gene were detected by DGGE but not by SSCP. Large rearrangements of the *TSC2* gene were identified by FISH and/or Southern blotting in 20 patients, accounting for 7% of the *TSC2* mutations. Of these, three were complete gene deletions, 12 were on one end of the gene, four were intragenic and one was an intragenic duplication. In 50% of cases the rearrangement was detected by both techniques.

Recurrent mutations were identified at both loci. The most common mutation was the 1850G>A (R611Q) missense mutation in exon 16 of the *TSC2* gene which was detected in 10 unrelated patients and, together with the adjacent 1849C>T (R611W) mutation that was detected in an additional four cases, accounted for 3.8% of the mutations identified. The most frequently occurring *TSC1* mutation, 1719C>T (R500X), was detected in six cases (1.7% of the total mutations).

The distributions of small mutations within the *TSC1* and *TSC2* genes are shown in Figure 2. The most mutations in the *TSC1* gene were identified in exon 15 (19/362; 5.2% of total mutations) and in the *TSC2* gene in exon 32 (32/362; 8.8% of total mutations). No mutations were identified in exons 3, 19, 22 and 23 of the TSC1 gene or in exon 30B of the *TSC2* gene. We calculated the mean number of mutations (including splice site changes) per nucleotide for each exon of both genes. The overall mutation frequency was higher at the TSC2 locus (0.045 mutations per nucleotide) than at the TSC1 locus (0.020 mutations per nucleotide). However, as shown in Figure 2, there was considerable variation in the frequency of mutations per exon. Exons 16 and 39 of the *TSC2* gene had the highest frequency of mutations.

Exons 35–39 of the *TSC2* gene encode the GTPase activating-protein (GAP) domain which is essential for tuberin function. We identified 64 small nucleotide mutations within these five exons, accounting for almost 18% of all the mutations identified. Interestingly, 46% of all the missense mutations and 93% of all the in-frame deletion mutations identified in the *TSC2* gene were in this region.

Phenotypic comparisons

We obtained clinical information on 291 patients, of which 276 (95%) fulfilled the definite TSC diagnostic criteria. In these 276 patients we identified 235 mutations; 53 in the *TSC1* gene and 182 in the *TSC2* gene, a mutation detection rate of 85%. In 15 individuals where there was insufficient clinical evidence for a definite diagnosis of TSC, we identified four mutations. In two cases mutations: *TSC1* 903C>T (R228X) and *TSC2* 4324delC), the patients were mentally retarded, had seizures, radiological brain abnormalities and hypomelanotic macules; in one case (mutation *TSC2* 1135C>T (Q373X)) dental pits and facial angiofibroma were noted in combination with epilepsy and mental retardation; and in one case (mutation *TSC2* 4324C>T (R1436X)), subependymal nodules were the only clinical feature noted.

We compared patients with a *TSC1* mutation to those with a *TSC2* mutation (Tables 3a and

3b). The median age of the patients with a *TSC1* mutation was not significantly different from the median age of those with a *TSC2* mutation (P=0.321). However, individuals with a *TSC1* mutation were less often mentally retarded (P<0.001), and renal angiomyolipomas (P<0.001), renal cysts (P=0.050), retinal phakomas (P=0.003) and retinal depigmentations (P=0.073) were all less frequent in the *TSC1* mutation group. In contrast, shagreen patches occurred significantly more frequently in patients with a *TSC1* mutation (P=0.031). Interestingly, although the incidence of mental retardation was lower in the group of patients with a *TSC1* mutation, the incidences of seizures (P=0.595) and (sub)cortical tubers (P=0.299) were not significantly different between the two groups.

We compared the phenotypes of individuals with a *TSC2* (i) nonsense or frameshift mutation; (ii) missense mutation; (iii) large abnormality; and (iv) mutation in exons 35–39 (encoding the GAP domain). Both renal angiomyolipomas and renal cysts were more frequent in the group with nonsense and frameshift mutations (64% and 37%, respectively) than in the group with missense mutations (38% and 24%, P=0.050 and 0.367), or mutations in the GAP domain (39% and 10%, P=0.032 and 0.018). Shagreen patches, forehead plaques, facial angiofibromas and ungual fibromas also occurred more often in the group of patients with a nonsense or frameshift mutation. The incidence of renal cysts was highest in the group of patients with large abnormalities of the *TSC2* gene (out of 10 patients with clinical information, three patients with a whole gene deletion and one patient with an intragenic duplication had renal cysts). Mental retardation, seizures and subependymal nodules occurred most frequently in the group of patients with a mutation in the GAP domain.

Next, we compared the clinical features in the 50 familial cases to the clinical features in the 91 individuals with a *de novo* mutation (Tables 3a and 3b). The median age was higher in the familial cases (P=0.226) and many clinical manifestations were less frequent. For subependymal nodules (P=0.001) and retinal phakomas (P=0.030) the differences were significant. In contrast, hypomelanotic macules were significantly more frequent in the familial cases (P=0.034).

In our cohort, *TSC2* mutations were associated with a more severe phenotypic spectrum than *TSC1* mutations (see above and Tables 3a and 3b). In addition, the proportion of *de novo* cases with a *TSC2* mutation (115/137; 84%) was higher than the proportion of familial cases with a *TSC2* mutation (43/63; 68%) (P=0.125), consistent with the more severe phenotypic spectrum observed in the *de novo* group. We compared the clinical features of familial and *de novo* cases with either a *TSC1* or a *TSC2* mutation. As shown in Tables 3a and 3b, 14 out of 17 clinical features were more frequent in the group of patients with a *de novo TSC2* mutation, than in the group with a familial *TSC2* mutation. Seizures and subependymal nodules occurred significantly more frequently in the *de novo* group (P=0.013 and P<0.001, respectively). The median age at referral was not significantly different between patients with a *de novo TSC2* mutation and familial cases (P=0.921). In contrast, patients with a familial *TSC1* mutation were referred for DNA testing at a significantly older age than patients with a *de novo TSC1* mutation (P=0.027). Subependymal nodules, forehead plaques, retinal phakoma and cardiac rhabdomyoma were more frequent in the group with a *de novo TSC1* mutation compared to the group with a familial *TSC1* mutation.

	TSC1						*TSC2*					
	Total		**familial**		***de novo***		**Total**		**familial**		***de novo***	
	P/N	**%**	**P/N**	**%**	**P/N**	**%**	**P/N**	**%**	**P/N**	**%**	**P/N**	**%**
Mental retardation	19/39	49	8/12	67	7/12	58	129/156	83	20/27	74	53/63	84
Seizures	42/46	91	13/13	100	13/15	87	159/175	91	28/34	82	69/71	97
Cortical tubers	20/24	83	6/6	100	7/8	88	93/104	89	14/18	78	37/39	95
SEN	34/37	92	10/11	91	11/12	92	125/138	91	16/22	73	58/58	100
SEGA	5/13	39	2/3	67	1/4	25	17/58	29	2/6	33	7/19	37
Renal AML	2/27	7	0/5		0/11		57/114	50	10/23	44	19/51	37
Renal cysts	3/28	11	1/5	20	0/11		27/98	28	5/20	25	12/44	27
Shagreen patch	22/31	71	7/9	78	5/9	56	42/85	49	3/11	27	22/39	56
Hypomelanotic macules	41/44	93	12/12	100	15/17	88	134/146	92	30/30	100	60/66	91
Forehead plaque	6/22	27	2/8	25	2/6	33	35/78	45	3/13	23	15/31	48
Facial angiofibromas	27/38	71	8/12	67	4/8	50	111/135	82	17/22	77	43/55	78
Ungual fibroma	14/31	45	2/8	25	1/6	17	30/89	34	4/15	27	15/39	39
Retinal phakoma	3/30	10	0/9		2/11	18	34/92	37	4/21	19	16/41	39
Retinal depigmentation	0/8		0/1		0/2		11/35	31	1/4	25	6/17	35
Gingival fibroma	5/10	50	2/2	100	1/2	50	10/36	28	0/1		6/19	32
Dental pits	10/19	53	2/4	50	2/5	40	27/54	50	4/7	57	14/27	52
Cardiac rhabdomyoma	10/24	42	1/3	33	6/11	55	37/89	42	7/17	41	17/39	44

Next, we compared the clinical features in the group of patients with a *de novo TSC1* mutation to the group with a *de novo TSC2* mutation. The majority of clinical features (14/17) were seen at a lower frequency in the group with a *de novo TSC1* mutation. Notably, mental retardation (P=0.055), renal angiomyolipomas (P=0.011) and renal cysts (P=0.048) were less frequent in the group with a *de novo TSC1* mutation. The median age at referral was not significantly different (P=0.364).

The phenotypic differences between the familial cases with *TSC1* and *TSC2* mutations were less pronounced. The only significant difference was the higher frequency of shagreen patches in the group with *TSC1* mutations (P=0.035). The median age at referral was not significantly different between these two groups (P=0.119).

Mutations Total		familial		*de novo*		No Mutation Identified	
P/N	%	P/N	%	P/N	%	P/N	%
148/195	76	28/39	72	60/75	80	8/22	36
201/221	91	41/47	87	82/86	95	17/25	68
113/128	88	20/24	83	44/47	94	14/18	78
159/175	91	26/33	79	69/70	99	18/22	82
22/71	31	4/9	44	8/23	35	1/14	7
59/141	42	10/28	36	19/62	31	9/16	56
30/126	24	6/25	24	12/55	22	5/16	31
64/116	55	10/20	50	27/48	56	3/16	19
175/190	92	42/42	100	75/83	90	17/23	74
41/100	41	5/21	24	17/37	46	2/15	13
138/173	80	25/34	74	47/63	75	14/22	64
44/120	37	6/23	26	16/45	36	4/15	27
37/122	30	4/30	13	18/52	35	2/16	13
11/43	26	1/5	20	6/19	32	1/10	10
15/46	33	2/3	67	7/21	33	2/5	40
37/73	51	6/11	55	16/32	50	1/4	25
47/113	42	8/20	40	23/50	46	5/15	33

Table 3a: Comparison of the clinical features in TSC patients with and without mutations. Familial represents the index patients of familial cases. For each group, the first column shows the number of patients with the feature (P) and the total number of patients examined (N) and the second column shows the frequency of each clinical feature (%). The median age was 14.0 for *TSC1* familial, 4.0 for *TSC1 de novo*, 15.0 for *TSC1* total, 11.0 for *TSC2* familial, 10.0 for *TSC2 de novo*, 12.0 for *TSC2* total, 13.0 for mutations total, 9.5 for *de novo*, 11.5 for familial and 12.0 for no mutation identified groups. (SEN; subependymal nodules, SEGA; subependymal giant cell astrocytoma, AML; angiomyolipoma.)

The clinical manifestations in 27 individuals with clinically definite TSC, without an identified pathogenic mutation, were compared to patients with definite TSC and an identified pathogenic mutation (Tables 3a and 3b). Although the median age at referral in the two groups was not significantly different (P=0.795), many of the clinical features including mental retardation (P<0.001), seizures (P=0.001), shagreen patches (P=0.006), hypomelanotic macules (P=0.006) and forehead plaques (P=0.033) occurred at a significantly lower frequency in the group without an identified mutation. Other symptoms were also observed less frequently in this group, although the differences were not statistically significant (p>0.05). Since the group of patients with a *TSC1* mutation was also associated with a less severe phenotype, we compared the clinical features in the group without an identified mutation to the group with a *TSC1* mutation. Most clinical

	TSC1 vs *TSC2*	Familial vs *de novo*	*TSC2* familial vs *TSC2 de novo*	*TSC1 de novo* vs *TSC2 de novo*
Mental retardation	<0.001*	0.322	0.203	0.055
Seizures	0.595	0.090	0.013*	0.139
Cortical tubers	0.299	0.169	0.072	0.436
SEN	0.550	0.001*	<0.001*	0.171
SEGA	0.368	0.454	0.637	0.565
Renal AML	<0.001*	0.634	0.399	0.011*
Renal cysts	0.050*	0.829	0.553	0.048*
Shagreen patch	0.031*	0.637	0.085	0.623
Hypomelanotic macules	0.527	0.034*	0.098	0.519
Forehead plaque	0.107	0.095	0.110	0.413
Facial angiofibromas	0.102	0.908	0.576	0.104
Ungual fibroma	0.178	0.430	0.315	0.292
Retinal phakoma	0.003*	0.030*	0.094	0.177
Retinal depigmentation	0.073	0.538	0.593	0.456
Gingival fibroma	0.172	0.308	0.700	0.567
Dental pits	0.528	0.795	0.571	0.500
Cardiac rhabdomyoma	0.586	0.648	0.552	0.380
Median age	0.321	0.226	0.921	0.364

features (13/17) were less frequent in the no mutation group. In particular, seizures (P=0.016), shagreen patches (P=0.001) and hypomelanotic macules (P=0.037) occurred less frequently. In contrast, renal angiomyolipomas (P=0.001) and renal cysts (P=0.100) were more common in the no mutation group.

DISCUSSION

Using a combination of DGGE, SSCP, FISH and Southern analysis, a total of 362 mutations were identified in 490patients (74%); of these, 235 mutations were present in 276 patients fulfilling the TSC definitive diagnostic criteria (85%), comparable to earlier studies[9, 10, 19]. The detection rate dropped to 62% in the group of patients without any clinical information and to 27% in patients where the clinical evidence was not sufficient for a definitive diagnosis. This suggests that our cohort contains individuals that have been misdiagnosed with TSC and that, in many patients,

TSC1 familial vs *TSC2* familial	*TSC1* familial vs *TSC1 de novo*	No mutation vs Mutations total	No mutation vs *TSC1* Total
0.456	0.500	<0.001*	0.351
0.125	0.780	0.001*	0.016*
0.288	0.571	0.188	0.473
0.232	0.739	0.168	0.227
0.405	0.371	0.066	0.067
0.087	**	0.270	0.001*
0.657	0.313	0.515	0.100
0.035*	0.310	0.006*	0.001*
**	0.335	0.006*	0.037*
0.656	0.594	0.033*	0.277
0.390	0.388	0.086	0.552
0.666	0.615	0.324	0.190
0.218	0.289	0.113	0.576
0.800	**	0.273	0.556
0.333	0.500	0.546	0.573
0.652	0.643	0.317	0.329
0.656	0.500	0.541	0.603
0.119	0.027*	0.795	0.484

Table 3b: Significance (p) values of the comparisons made between the different groups depicted in table 3a. * Indicates statistically significant values. Familial represents the index patients of familial cases. (SEN; subependymal nodules, SEGA; subependymal giant cell astrocytoma, AML; angiomyolipoma.) ** Indicates where statistical analysis could not be performed since the patients in both groups were either all positive or all negative for the analysed feature.

good clinical work-up will be sufficient to make a definite diagnosis of TSC. However, in some cases, molecular genetic screening of patients with an incomplete clinical evaluation can assist in establishing the diagnosis.

We identified significant correlations between the presence of mental retardation and seizures and the presence of (sub)cortical tubers and mental retardation similar to previous studies[10]. In addition, we identified significant correlations between the presence of seizures and (sub)cortical tubers, and between the presence of (sub)cortical tubers and subependymal nodules. Consistent with earlier work, we found a correlation between the incidence of renal angiomyolipomas and mental retardation in patients above 6 years of age[10, 20].

In our cohort, males with TSC were more likely to be mentally retarded and to have renal cysts, retinal lesions, facial angiofibromas, and gingival and ungual fibromas. Sex-linked differences in TSC-associated features have been described previously in humans and in animal models of

the disease. For example, males with TSC have a greater risk of learning disorders and autism than females with the disease[21]. In addition, sex hormones have been shown to affect the behaviour of cells derived from TSC-associated lesions[22] and in a mouse model of TSC, liver hemangiomas were more common and more extensive in female than in male mice[23]. It will be interesting to investigate in more detail how sex hormones regulate the functions of tuberin and hamartin, and to determine how hormonal changes influence the TSC phenotype.

Previous studies of large numbers of TSC patients have used denaturing high-performance liquid chromatography (DHPLC) for the detection of small nucleotide changes in the *TSC1* and *TSC2* genes[10, 19]. Although we used a different approach, we detected a similar spectrum of small *TSC1* and *TSC2* mutations. In 94 cases where both SSCP and DGGE were performed and a mutation was identified, DGGE had a slightly higher mutation detection rate (93%) than SSCP (84%). For various technical reasons, a small number of mutations at the TSC1 and TSC2 loci are likely to be missed by either SSCP or DGGE. Due to its higher capacity and sensitivity, DGGE is now the method of choice in our laboratory. Exons not amenable to DGGE analysis, are directly sequenced. To detect larger rearrangements at the TSC2 locus, we performed FISH and Southern blotting. Neither technique alone detected all of the rearrangements in our cohort and therefore we are currently developing a multiplex ligation-dependent probe amplification assay (MLPA).

We found a similar predominance of *TSC2* mutations in both *de novo* and familial cases as previous studies[9-11, 24]. We also confirmed the lower frequency of *de novo TSC1* mutations compared to *de novo TSC2* mutations, which has been attributed to the smaller size and less complex structure of the TSC1 genomic locus, and the rarity of large DNA rearrangements, missense and splice site mutations at this locus[24]. It has also been suggested that *TSC1* mutations are associated with a less severe TSC phenotype[9, 10]. In our cohort, individuals with a *TSC1* mutation were less likely to be mentally retarded than patients with a *TSC2* mutation, even though the incidence of tubers and seizures in the group of patients with a *TSC1* mutation was not significantly lower. Previous studies have suggested that there is a correlation between tuber number and mental retardation in TSC patients and that, on average, fewer tubers and subependymal nodules are found in patients with a *TSC1* mutation[10]. We were unable to obtain data on the number of tubers to determine whether this could explain the lower incidence of mental retardation in the patients with a *TSC1* mutation in our cohort. Renal and retinal abnormalities occurred less frequently in the group of patients with a *TSC1* mutation, consistent with other studies[9, 10]. Although the incidence of dermatological abnormalities was not significantly reduced in this group, shagreen patches were present significantly more often. The lower incidence of renal abnormalities in this group, in contrast to the approximately equal incidence of brain and skin lesions, is consistent with the proposal that at least some of the phenotypic differences between these two groups is due to a differential role of tuberin and hamartin in renal cell growth control[25].

We identified a lower proportion of *TSC2* mutations in familial cases of TSC than in *de novo* cases (68 vs 84%). We compared the clinical features of both familial and *de novo* cases of TSC caused by mutations to either gene. Similar to the comparison of the cohort as a whole (see

above), *de novo TSC1* mutations were associated with a lower frequency of clinical features than *de novo TSC2* mutations. In familial cases, however, there was less difference between the clinical features of the groups of patients with a *TSC1* or *TSC2* mutation. This suggested that familial cases with a TSC2 mutation are associated with a milder TSC phenotype than *de novo* TSC2 cases, which was supported by a comparison of the frequencies of their clinical features. We did not detect any significant phenotypic differences between familial and *de novo* cases with a *TSC1* mutation.

The mutation frequency, as calculated per exonic nucleotide, at the TSC2 locus was higher compared to that at the TSC1 locus and, not surprisingly, the exons with the highest frequencies of mutation (*TSC2* exons 16 and 39) both contained recurrent mutations. The high proportion of nontruncating mutations in exons 35–39 of the *TSC2* gene supports previous findings that the GAP domain of the *TSC2* gene is a target for missense mutations[26].

The frequencies of neurological symptoms and some brain lesions were slightly higher and the incidence of renal abnormalities was lower in patients with mutations in exons 35–39 than in other groups. These differences were not significant. Overall, we could not confirm that mutations to the tuberin GAP domain are associated with a more severe phenotype[26]. The high incidence of renal cysts in the group with large abnormalities is consistent with the disruption of the adjacent PKD1 gene in several of these cases[1].

In 27 patients with definite TSC, we did not identify a mutation in either the *TSC1* or *TSC2* gene. Most clinical features, with the exception of renal abnormalities and gingival fibromas, occurred less frequently in this group of patients compared to the group with a mutation. It is possible that mutations in regulatory regions that impair gene transcription or translation account for the milder phenotypes of these cases. Somatic mosaicism may also explain why in some patients no mutations were detected[10]. Recently, Roberts et al[27] reported somatic mosaicism in 3% of patients diagnosed with definite TSC. We have identified eight cases of somatic mosaicism: only one patient and seven parents of a patient. Since we stopped using an allele specific method for family studies, it is possible that some mosaic parents have been missed. It is likely that there are more mosaic patients in our cohort with a degree of mosaicism below the detection limit of the current screening protocol.

In summary, in our diagnostic setting of TSC mutation analysis an overall mutation detection rate of 74% was obtained; a pathogenic mutation was identified in 85% of definite TSC patients. Our study reveals that several clinical manifestations of TSC occur more frequently in males. In addition, *TSC2* mutations are associated with a more severe phenotype than *TSC1* mutations, and the group of patients with a *de novo TSC2* mutation have a more severe phenotypic spectrum than the group of patients with a familial *TSC2* mutation. The identification of a *TSC1* or a *TSC2* mutation will not only confirm the diagnosis of TSC but will also be important for both clinical management and possible therapeutic or preventive measures.

ACKNOWLEDGEMENTS

We thank all the patients who contributed DNA samples for analysis, and the referring clinicians. Gregory Asmawidjaja, Anita Jankie, Kim van Zijl, Rodney Blenham and Margje van Schaik are thanked for technical assistance. This work was supported by the Nationaal Epilepsie Fonds, Noortman BV, the Nederlandse Organisatie voor Wetenschappelijk Onderzoek and the Department of Clinical Genetics at the Erasmus MC.

REFERENCES

1. Gomez, M., J. Sampson, and V. Whittemore, *The Tuberous Sclerosis Complex*. 1999, Oxford, England: Oxford University Press.
2. Henske, E.P., et al., *Allelic loss is frequent in tuberous sclerosis kidney lesions but rare in brain lesions.* Am J Hum Genet, 1996. **59**(2): p. 400-6.
3. Nellist, M., et al., *Characterization of the cytosolic tuberin-hamartin complex. Tuberin is a cytosolic chaperone for hamartin.* J Biol Chem, 1999. **274**(50): p. 35647-52.
4. Garami, A., et al., *Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2.* Mol Cell, 2003. **11**(6): p. 1457-66.
5. *Identification and characterization of the tuberous sclerosis gene on chromosome 16. The European Chromosome 16 Tuberous Sclerosis Consortium.* Cell, 1993. **75**(7): p. 1305-15.
6. van Slegtenhorst, M., et al., *Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34.* Science, 1997. **277**(5327): p. 805-8.
7. van Slegtenhorst, M., et al., *Mutational spectrum of the TSC1 gene in a cohort of 225 tuberous sclerosis complex patients: no evidence for genotype-phenotype correlation.* J Med Genet, 1999. **36**(4): p. 285-9.
8. Au, K.S., et al., *Germ-line mutational analysis of the TSC2 gene in 90 tuberous-sclerosis patients.* Am J Hum Genet, 1998. **62**(2): p. 286-94.
9. Jones, A.C., et al., *Comprehensive mutation analysis of TSC1 and TSC2-and phenotypic correlations in 150 families with tuberous sclerosis.* Am J Hum Genet, 1999. **64**(5): p. 1305-15.
10. Dabora, S.L., et al., *Mutational analysis in a cohort of 224 tuberous sclerosis patients indicates increased severity of TSC2, compared with TSC1, disease in multiple organs.* Am J Hum Genet, 2001. **68**(1): p. 64-80.
11. Niida, Y., et al., *Analysis of both TSC1 and TSC2 for germline mutations in 126 unrelated patients with tuberous sclerosis.* Hum Mutat, 1999. **14**(5): p. 412-22.
12. Langkau, N., et al., *TSC1 and TSC2 mutations in tuberous sclerosis, the associated phenotypes and a model to explain observed TSC1/ TSC2 frequency ratios.* Eur J Pediatr, 2002. **161**(7): p. 393-402.
13. Cheadle, J.P., et al., *Molecular genetic advances in tuberous sclerosis.* Hum Genet, 2000. **107**(2): p. 97-114.

14. Verhoef, S., et al., *High rate of mosaicism in tuberous sclerosis complex.* Am J Hum Genet, 1999. **64**(6): p. 1632-7.
15. Eussen, B.H., et al., *An unbalanced submicroscopic translocation t(8;16)(q24.3;p13.3)pat associated with tuberous sclerosis complex, adult polycystic kidney disease, and hypomelanosis of Ito.* J Med Genet, 2000. **37**(4): p. 287-91.
16. Nellist, M., et al., *TSC2 missense mutations inhibit tuberin phosphorylation and prevent formation of the tuberin-hamartin complex.* Hum Mol Genet, 2001. **10**(25): p. 2889-98.
17. Nellist, M., et al., *Distinct effects of single amino-acid changes to tuberin on the function of the tuberin-hamartin complex.* Eur J Hum Genet, 2005. **13**(1): p. 59-68.
18. Roach, E.S., et al., *Tuberous Sclerosis Consensus Conference: recommendations for diagnostic evaluation. National Tuberous Sclerosis Association.* J Child Neurol, 1999. **14**(6): p. 401-7.
19. Jones, A.C., et al., *Application and evaluation of denaturing HPLC for molecular genetic analysis in tuberous sclerosis.* Hum Genet, 2000. **106**(6): p. 663-8.
20. O'Callaghan, F.J., M. Noakes, and J.P. Osborne, *Renal angiomyolipomata and learning difficulty in tuberous sclerosis complex.* J Med Genet, 2000. **37**(2): p. 156-7.
21. Smalley, S.L., et al., *Autism and tuberous sclerosis.* J Autism Dev Disord, 1992. **22**(3): p. 339-55.
22. Yu, J., et al., *Estradiol and Tamoxifen Stimulate Lymphangiomyomatosis-Associated Angiomyolipoma Cell Growth and Activate Both Genomic and Non-Genomic Signaling Pathways.* Am J Physiol Lung Cell Mol Physiol, 2003.
23. Kwiatkowski, D.J., et al., *A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells.* Hum Mol Genet, 2002. **11**(5): p. 525-34.
24. Kwiatkowski, D.J., *Tuberous sclerosis: from tubers to mTOR.* Ann Hum Genet, 2003. **67**(Pt 1): p. 87-96.
25. Yeung, R.S., *Multiple roles of the tuberous sclerosis complex genes.* Genes Chromosomes Cancer, 2003. **38**(4): p. 368-75.
26. Maheshwar, M.M., et al., *The GAP-related domain of tuberin, the product of the TSC2 gene, is a target for missense mutations in tuberous sclerosis.* Hum Mol Genet, 1997. **6**(11): p. 1991-6.
27. Roberts, P.S., et al., *Somatic mosaicism is rare in unaffected parents of patients with sporadic tuberous sclerosis.* J Med Genet, 2004. **41**(5): p. e69.

LARGE DELETION AT THE *TSC1* LOCUS IN A FAMILY WITH TUBEROUS SCLEROSIS COMPLEX

M. Nellist[1], O. Sancak[1], M.A. Goedbloed[1], M. van Veghel-Plandsoen[1], A. Maat-Kievit[1], D. Lindhout[2], B.H. Eussen[1], A. de Klein[1], D.J.J. Halley[1] and A.M.W. van den Ouweland*[1].

[1] Department of Clinical Genetics, Erasmus Medical Center, 3015 GE Rotterdam, The Netherlands.
[2] Department of Medical Genetics, Utrecht University Medical Center, 3508 AB Utrecht, The Netherlands.

Running title: Large *TSC1* deletion

In press: Genetic Testing

*Correspondence: Dr A van den Ouweland,
Department of Clinical Genetics,
Erasmus Medical Center,
Dr Molewaterplein 50,
3015 GE Rotterdam, The Netherlands.
Tel: +31 10 4087197;
Fax: +31 10 4089489;
E-mail: a.vandenouweland@erasmusmc.nl

ABSTRACT

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by seizures, mental retardation and the development of hamartomas in a variety of organs and tissues. The disease is caused by mutations in either the *TSC1* gene on chromosome 9q34, or the *TSC2* gene on chromosome 16p13.3. Here we describe a deletion encompassing the *TSC1* gene and two neighbouring transcripts on chromosome 9q34 in 6 affected individuals from a family with TSC. To our knowledge, this is the first report of such a large deletion at the *TSC1* locus and indicates that screening for similar mutations at the *TSC1* locus is warranted in individuals with TSC.

INTRODUCTION

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by seizures, mental retardation and the development of hamartomas in a variety of organs and tissues. The disease is caused by mutations in either the *TSC1* gene on chromosome 9q34, or the *TSC2* gene on chromosome 16p13.3. Loss of heterozygosity studies at the *TSC1* and *TSC2* loci in TSC-associated lesions indicate that *TSC1* and *TSC2* are tumour suppressor genes [1].

The *TSC1* gene consists of 23 exons that span 50 kb of genomic DNA. The 3.4 kb open reading frame of the 8.5 kb *TSC1* mRNA encodes a 1,164 amino acid protein called hamartin. The *TSC2* gene consists of 41 exons that span 40 kb of genomic DNA, and the 5.5 kb *TSC2* mRNA encodes a 1,807 amino acid protein called tuberin. Hamartin and tuberin form a complex that directly activates the GTPase activity of rheb, and thereby prevents the rheb-GTP-dependent stimulation of cell growth through mTOR [2].

In four different studies of large cohorts of TSC patients, multi-exon and whole gene deletions, involving up to several hundred kilobases (kb) of genomic DNA, accounted for 17% (78/452) of all the mutations identified at the *TSC2* locus [3-6]. Methods such as pulsed field gel electrophoresis, fluoresence in situ hybridisation (FISH), Southern blotting and long-range or quantitative PCR are therefore essential for mutation screening of the *TSC2* gene. In the same four studies, only 3 multi-exon deletions at the *TSC1* locus were identified, accounting for 2.8% (3/107) of the TSC1 mutations identified. In each of these cases, the deletion involved less than 10 kb of genomic DNA and did not affect any of the genes adjacent to the *TSC1* locus [5]. Here we describe a large kindred segregating TSC and a large (>40 kb) deletion affecting not only *TSC1* but also two neighbouring genes. To our knowledge this is the first example of a multi-gene deletion at the *TSC1* locus and indicates that screening for large deletions at the *TSC1* locus is warranted in individuals with TSC.

MATERIALS AND METHODS

DNA was extracted from peripheral blood cells using standard techniques. Linkage analysis was performed using microsatellite markers spanning the *TSC1* and *TSC2* loci, as described previously [7]. The *TSC1* gene was screened for mutations using a combination of single-

strand conformational polymorphism analysis, denaturing gradient gel electrophoresis, direct sequencing and Southern blotting [6]. Single nucleotide polymorphisms were amplified by PCR (primers available on request) and typed by sequencing. FISH was performed on cultured skin fibroblasts following a standard protocol [8] and the exact extent of the deletion was determined by PCR, followed by sequencing.

RESULTS

Clinical investigations

The pedigree is shown in Figure 1 and the results of the clinical investigations are summarised in Table 1. Individuals II-2, II-4, II-5, III-2, III-3 and III-4 fulfilled the criteria for a definitive diagnosis of TSC. Individuals II-3, II-6, II-7 and III-6 were investigated but did not have any signs of TSC. Individuals II-1, II-8, II-9, II-10, II-11, II-12, III-1, III-5, III-7, III-8 and III-9 were not available for a detailed clinical assessment but had no medical history of TSC.

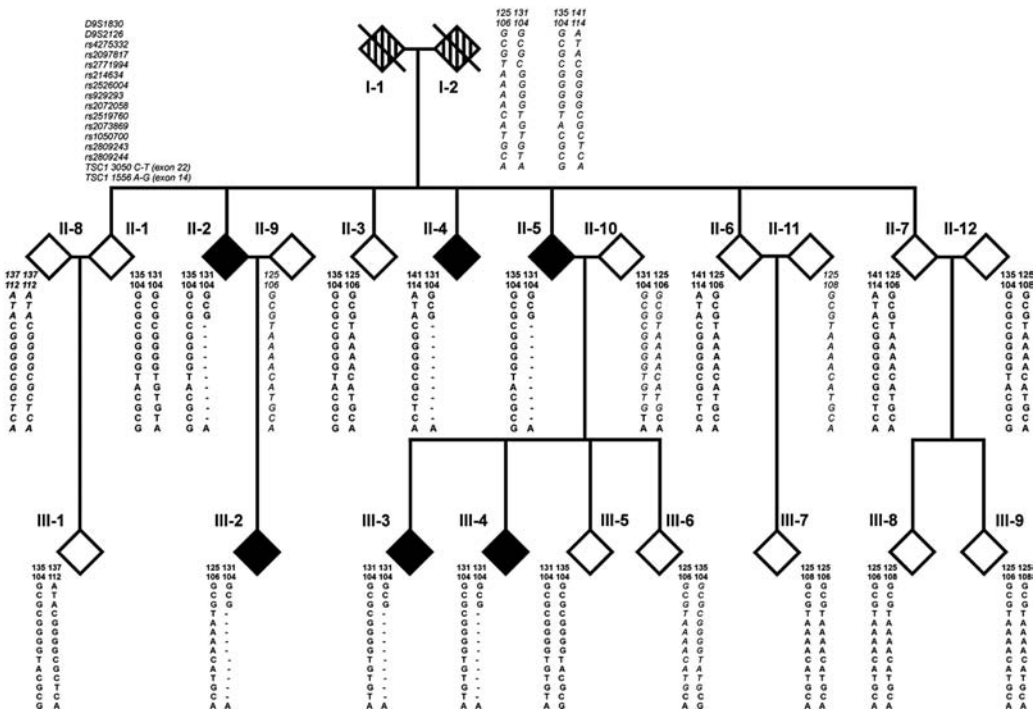


Figure 1. Haplotypes for markers extending distal from the *TSC1* locus in a chromosome 9q34-linked family with TSC. The alleles for the intragenic *TSC1* polymorphisms 1556A-G and 3050C-T, 12 SNPs and the microsatellite markers D9S2126 and D9S1830 are indicated for each individual from distal (top) to proximal (bottom). Inferred haplotypes are in italics.

TSC features	II.2	II.4	II.5	III.2	III.3	III.4
Facial angiofibroma/ forehead plaque	+	+	+	+	+	+
Ungual fibroma	+	+	+	+	+	+
Hypomelanotic maculae	+			+	+	+
Shagreen patch	+	+	+	+	+	+
Cortical tuber		+	+			
Subependymal nodule	+	+	+	+	+	+
Subependymal giant cell astrocytoma					+	
Dental pits	+		+		+	
Bone cysts	+	+				
Gingival fibroma	+				+	
Epilepsy	+		+	+	+	+
Mental retardation				+	+	+

Table 1. Results of the clinical investigations. The diagnostic signs of TSC present in the affected individuals in the family are indicated.

Mutation analysis

There was no evidence for linkage to the *TSC2* locus in the family (LOD score = 0.00). However, the LOD score for markers surrounding the *TSC1* locus was suggestive for linkage (maximum LOD = 1.81). Imaginary chromosome analysis indicated that TSC was genetically linked to the *TSC1* locus (probability >0.999; Janssen et al., 1994) and a comprehensive mutation screen of the *TSC1* gene was performed. No pathogenic nucleotide changes or abnormal restriction fragments were detected. However, several informative intragenic polymorphisms were identified and one of these, *TSC1* 3050 C-T in exon 22, showed an inheritance pattern consistent with the presence of a deletion in the affected individuals in the pedigree. Subsequently, 12 single nucleotide polymorphisms (SNPs) mapping close to the *TSC1* locus (rs4275332, rs2097817, rs2771994, rs214634, rs2526004, rs929293, rs2072058, rs2519760, rs2073869, rs1050700, rs2809243 and rs2809244) were typed (Figure 1). All the affected individuals were hemizygous across a region extending from exon 22 until at least 36 kb distal of the *TSC1* gene. The presence of a deletion was confirmed by FISH analysis in fibroblasts derived from individual II-5 using cosmids 115B2 and 250B9 that map to the 3' end of the *TSC1* gene [9] (Figure 2A), and by PCR amplification of the deletion breakpoint fragment in all the affected individuals (not shown). To identify the deletion breakpoint, patient DNA was amplified by PCR using one primer mapping just 5' to exon 20 of the *TSC1* gene in combination with a series of primers mapping at appoximately 2 kb intervals between rs2771994 and rs214634. Sequence analysis of the breakpoint fragment indicated that the deletion encompassed 43 kb (42 985 nucleotides), extending from intron 20 of the *TSC1* gene to intron 4 of the c9orf98 gene and encompassing the complete c9orf9 (Figure 2B and C).

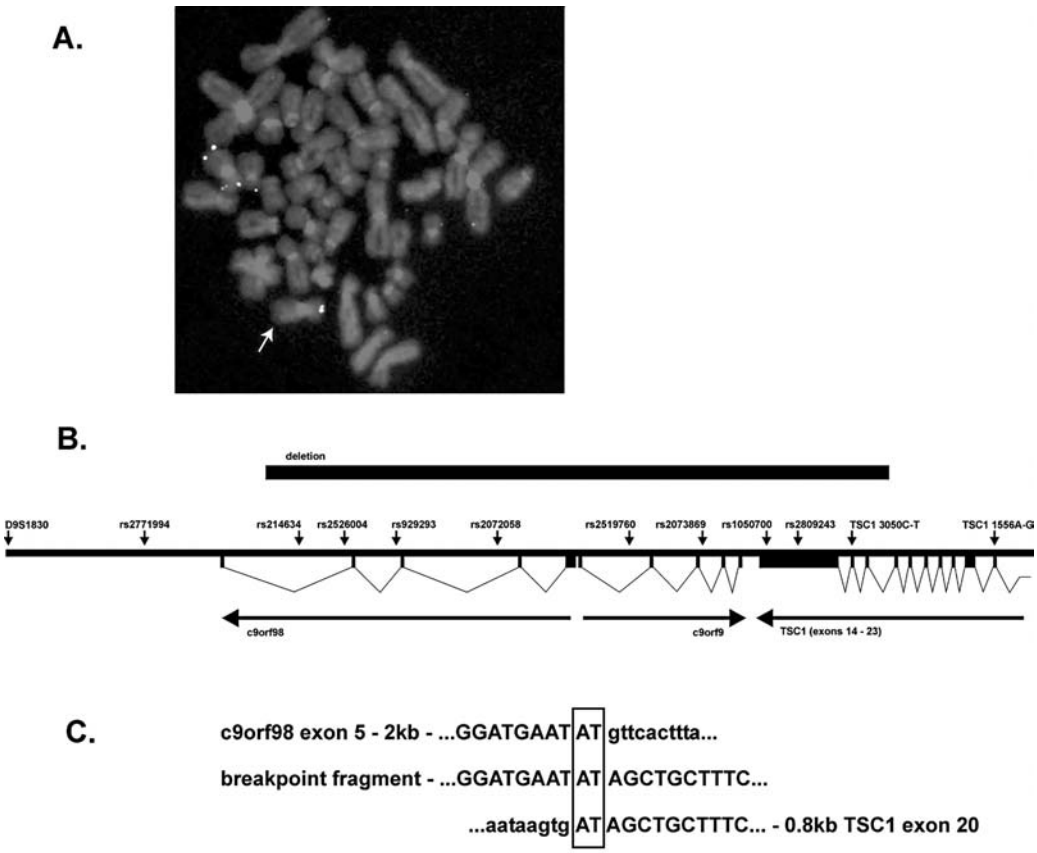


Figure 2. Characterisation of the deletion. (A) FISH analysis of individual II-5 with *TSC1* cosmid clone 250B9 (labelled in green) and the control 9p telomere probe RP1-43N6 (labelled in red). The abnormal chromosome 9, lacking the *TSC1* signal, is indicated with an arrow. (B) Schematic diagram indicating the genomic extent of the deletion. The region of chromosome 9q34 investigated as part of this study, extending from the distal microsatellite marker D9S1830 (left) to the proximal intragenic *TSC1* 1556A-G polymorphism (right) is shown. Individual exons, and all the markers tested as part of this study are indicated. Horizontal arrows indicate the extent and orientation of the *TSC1*, c9orf9 and c9orf98 transcripts. The extent of the deletion is indicated by the solid bar. (C) Sequence across the deletion breakpoint. The *TSC1* intron 20 and c9orf98 intron 4 sequences in the region of both deletion breakpoints are shown, as well as the breakpoint sequence in the affected individuals.

DISCUSSION

The identification of the *TSC2* gene was facilitated by the presence of multi-exon and whole gene deletions across the *TSC2* locus [10]. It has been suggested that the absence of similar large deletions at the *TSC1* locus may reflect selection against deletions involving genes adjacent to *TSC1* [11] and, perhaps as a consequence, not all studies of mutations in TSC patients have screened for large deletions at the *TSC1* locus [4]. Here we report a large deletion that not only

inactivates the *TSC1* gene, but also disrupts two neighbouring genes, c9orf9 and c9orf98. c9orf9 lies in a tail to tail orientation with *TSC1* and encodes a homolog of rsb-66, a small protein that is expressed specifically in the testis [12]. c9orf98 lies distal to c9orf9, in the same orientation as *TSC1*, and encodes a putative adenylate kinase.

Comparison of large numbers of TSC patients with known *TSC1* or *TSC2* mutations indicates that, overall, *TSC1* mutations are associated with less severe disease [4]. However, individuals with a *TSC1* mutation can still be severely affected. The individuals in this family were clearly affected with TSC. Interestingly, no renal symptoms were noted (see Table 1), in line with the finding that renal lesions occur less often in patients with a *TSC1* mutation than in patients with a *TSC2* mutation [6]. Although the precise functions of the c9orf9 and c9orf98 protein products are still unclear, the phenotypes of the affected individuals in this kindred suggest that loss of either gene does not severely aggravate the TSC phenotype.

Previously, Longa et al. reported three intragenic *TSC1* deletions [5]. These deletions were significantly smaller than the mutation described here and did not affect the neighbouring gene loci. In our case, as well as those described by Longa et al, the deletion breakpoints involved sequences in intron 20 of the *TSC1* gene. This intron contains a long terminal repeat (LTR) class repetitive element. Interestingly, a cluster of similar repeats in intron 4 of the c9orf98 gene lie close to the breakpoint in our family and, although it is not clear how the deletion arose, one possibility is that the LTR-class repeats were involved.

Closer inspection of the haplotypes in the pedigree indicated that individual II-1 had inherited the same chromosome 9 haplotype as the affected individuals II-2, II-4 and II-5, but was heterozygous for the *TSC1* 3050 C-T polymorphism and the rs1050700 and rs2809243 SNPs. Although individual II-1 suffered from epilepsy and hydrocephalus, cranial imaging studies indicated that this was not due to the presence of either subependymal nodules or a subependymal giant cell astrocytoma. We concluded that the 43 kb deletion was not present in individual II-1, and that the epilepsy and hydrocephalus in this individual was unlikely to be due to TSC. Our data indicate that one of the parents of II-1 (individual I-1 or I-2) was a germ-line mosaic for the 43 kb deletion identified in the affected individuals II-2, II-4, II-5, III-2, III-3 and III-4.

In previous work, no large deletions were identified at the *TSC1* locus in a cohort of 225 TSC patients screened by Southern blotting [13]. The case reported here is the only large *TSC1* deletion identified in this cohort. Most molecular diagnostic screens fail to identify *TSC1* or *TSC2* mutations in all the TSC patients tested [4, 6]. Therefore, large deletions at the *TSC1* locus may be under-represented partly because the appropriate diagnostic tests have not been performed. It is possible that there are more, as yet undetected, mutations in the TSC patient population that affect multiple exons of the *TSC1* gene.

The mutation in this family indicates that to detect all the possible mutations in TSC patients, it will be necessary to screen both the *TSC1* and *TSC2* loci for multi-exon and whole gene deletions. Screening for large deletions at the *TSC1* locus is therefore warranted in individuals with TSC.

ACKNOWLEDGEMENTS

The authors would like to thank the members of the family for providing samples and co-operating with this study. M. Klunnen is thanked for assistance with the FISH analysis. Financial support was provided by the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO), Noortman b.v. and the Department of Clinical Genetics (Erasmus MC).

REFERENCES

1. Sampson, J.R., *TSC1 and TSC2: genes that are mutated in the human genetic disorder tuberous sclerosis.* Biochem Soc Trans, 2003. **31**(Pt 3): p. 592-6.
2. Li, Y., et al., *TSC2: filling the GAP in the mTOR signaling pathway.* Trends Biochem Sci, 2004. **29**(1): p. 32-8.
3. Jones, A.C., et al., *Comprehensive mutation analysis of TSC1 and TSC2-and phenotypic correlations in 150 families with tuberous sclerosis.* Am J Hum Genet, 1999. **64**(5): p. 1305-15.
4. Dabora, S.L., et al., *Mutational analysis in a cohort of 224 tuberous sclerosis patients indicates increased severity of TSC2, compared with TSC1, disease in multiple organs.* Am J Hum Genet, 2001. **68**(1): p. 64-80.
5. Longa, L., et al., *TSC1 and TSC2 deletions differ in size, preference for recombinatorial sequences, and location within the gene.* Hum Genet, 2001. **108**(2): p. 156-66.
6. Sancak, O., et al., *Mutational analysis of the TSC1 and TSC2 genes in a diagnostic setting: genotype--phenotype correlations and comparison of diagnostic DNA techniques in Tuberous Sclerosis Complex.* Eur J Hum Genet, 2005. **13**(6): p. 731-41.
7. Janssen, B., et al., *Refined localization of TSC1 by combined analysis of 9q34 and 16p13 data in 14 tuberous sclerosis families.* Hum Genet, 1994. **94**(4): p. 437-40.
8. Eussen, B.H., et al., *An unbalanced submicroscopic translocation t(8;16)(q24.3;p13.3)pat associated with tuberous sclerosis complex, adult polycystic kidney disease, and hypomelanosis of Ito.* J Med Genet, 2000. **37**(4): p. 287-91.
9. Hornigold, N., et al., *A 1.7-megabase sequence-ready cosmid contig covering the TSC1 candidate region in 9q34.* Genomics, 1997. **41**(3): p. 385-9.
10. *Identification and characterization of the tuberous sclerosis gene on chromosome 16. The European Chromosome 16 Tuberous Sclerosis Consortium.* Cell, 1993. **75**(7): p. 1305-15.
11. van Slegtenhorst, M., et al., *Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34.* Science, 1997. **277**(5327): p. 805-8.
12. Yang, M., et al., *Crystallization and preliminary crystallographic analysis of RSB-66, a novel round spermatid-specific protein.* Acta Crystallogr D Biol Crystallogr, 2003. **59**(Pt 10): p. 1853-5.
13. van Slegtenhorst, M., et al., *Mutational spectrum of the TSC1 gene in a cohort of 225 tuberous sclerosis complex patients: no evidence for genotype-phenotype correlation.* J Med Genet, 1999. **36**(4): p. 285-9.

DISTINCT EFFECTS OF SINGLE AMINO-ACID CHANGES TO TUBERIN ON THE FUNCTION OF THE TUBERIN–HAMARTIN COMPLEX

Mark Nellist*[1], Ozgur Sancak[1], Miriam A Goedbloed[1], Christan Rohe[1], Diana van Netten[1], Karin Mayer[2], Aimee Tucker-Williams[3], Ans MW van den Ouweland[1] and Dicky JJ Halley[1]

[1]Department of Clinical Genetics, Erasmus MC, 3015 GE Rotterdam, The Netherlands; [2]Laboratory for Medical Genetics, Lochhamerstrasse 29, 82152 Martinsried, Germany; [3]Department of Pediatrics, University of Texas Medical School, Houston, TX, USA

European Journal of Human Genetics (2005) 13, 59–68

Received 27 May 2004; revised 22 July 2004; accepted 27 July 2004

European Journal of Human Genetics (2005) 13, 59–68. doi:10.1038/sj.ejhg.5201276
Published online 13 October 2004

*Correspondence: Dr M Nellist,
Department of Clinical Genetics,
Erasmus MC,
Dr. Molewaterplein 50,
3015 GE Rotterdam, The Netherlands.
Tel: +31 10 4087357;
Fax: +31 10 4089489;
E-mail: m.nellist@erasmusmc.nl

ABSTRACT

Tuberous sclerosis is an autosomal dominant human disorder caused by inactivating mutations to either the *TSC1* or *TSC2* tumour suppressor gene. Hamartin and tuberin, the *TSC1* and *TSC2* gene products, interact and the tuberin–hamartin complex inhibits cell growth by antagonising signal transduction to downstream effectors of the mammalian target of rapamycin (mTOR) through the small GTPase rheb. Previously, we showed that pathogenic tuberin amino-acid substitutions disrupt the tuberin–hamartin complex. Here, we investigate how these mutations affect the role of tuberin in the control of signal transduction through mTOR. Our data indicate that specific amino-acid substitutions have distinct effects on tuberin function.

Keywords: tuberin; hamartin; tuberous sclerosis

INTRODUCTION

Tuberous sclerosis (TSC) is an autosomal dominant disorder caused by inactivation of either the *TSC1* gene on chromosome 9q34 or the *TSC2* gene on chromosome 16p13.3[1, 2]. TSC is characterised by the development of hamartomata in many tissues and organs. Brain and skin involvement results in the classic phenotype of seizures, mental retardation, and facial and ungual fibromas[3].

The *TSC1* and *TSC2* gene products, hamartin and tuberin, interact to form a complex that inhibits cell growth by antagonising signal transduction through the mammalian target of rapamycin (mTOR)[4]. Inactivation of the tuberin–hamartin complex results in phosphorylation of the downstream effectors of mTOR, p70 S6 kinase (S6K), ribosomal protein S6 and the elongation factor binding protein 4E-BP1[5-7]. The tuberin–hamartin complex antagonises mTOR through activation of the GTPase activity of rheb[8, 9].

In a mutation screen of 490 TSC patients, 22% of the pathogenic changes we identified were truncating *TSC1* mutations (O Sancak, manuscript in preparation). We did not find any *TSC1* missense mutations. In contrast, *TSC2* missense mutations accounted for 15% of all the mutations detected in our cohort. In some cases, due to the unavailability of parental DNA and/or the lack of sufficient clinical data, we have not yet been able to determine whether specific missense changes are pathogenic. We are interested in characterising how missense changes affect tuberin function to help establish whether specific missense changes are pathogenic.

Previously, we found that the tuberin amino-acid substitutions R611Q, R611W, A614D, F615S, C696Y and V769E disrupted the interaction between tuberin and hamartin, destabilising the tuberin–hamartin complex and resulting in the loss of hamartin from preparations of cytosol. The R611Q, R611W, A614D, F615S, C696Y and V769E substitutions also prevented tuberin phosphorylation. In contrast, the N525S, K599M and R905Q substitutions did not affect the tuberin–hamartin interaction and did not prevent tuberin phosphorylation[10]. We compared the effects of these substitutions and six additional aminoacid changes (R367Q, A607T, 609insS,

L826M, P1202H and G1556S) on the tuberin–dependent phosphorylation of S6K and S6 and on the stimulation of the in vitro GTPase activity of rheb by tuberin.

Our analysis indicates that amino-acid changes have distinct effects on tuberin function. We show that some missense mutations outside the tuberin GAP domain completely inactivate the tuberin–hamartin complex, inhibiting the tuberin GAP activity and permitting phosphorylation of the downstream effectors of mTOR.

MATERIALS AND METHODS

Generation of constructs and antisera

Full-length *TSC1* and *TSC2* expression constructs have been described previously[11]. Truncated *TSC2* cDNAs encoding amino acids 1–1240 (Eco47III truncation), 1–1099 (XmaI truncation), 1–607 (NruI truncation), 607–1099 and 1125–1784 (NruI-XmaI fragments) and 1–252 plus 1536–1784 (SacI internal in-frame deletion) were cloned behind an amino-terminal polyhistidine epitope tag derived from the pQE series of vectors (Qiagen) in the pSG5 expression vector. Expression constructs encoding the R367Q, N525S, K599M, A607T, 609insS, R611Q, R611W, A614D, F615S, C696Y, V769E, L826M, R905Q, P1202H and G1556S tuberin variants were derived by site-directed mutagenesis using the Stratagene QuickChange kit. All constructs were sequenced completely. Amino-acid residues are numbered according to reference 2.

An expression construct for the production of GST-rheb in bacteria was provided by M van Slegtenhorst (Fox Chase Cancer Center, Philadelphia, USA) and the 2B4 construct, encoding S6K, was provided by T Nobokuni (Friedrich Miescher Institute, Basel, Switzerland). Expression constructs encoding activated and dominant-negative isoforms of PKB were purchased from Upstate Biotechnology. The rap1GAP expression construct was obtained from Invitrogen.

Polyclonal rabbit antisera specific for human tuberin and hamartin have been described previously[11]. Mouse monoclonal antibodies against the polyhistidine epitope tag were purchased from Qiagen. All other antibodies were purchased from Cell Signaling Technology.

Immunocytochemistry

The *Tsc1* -/- and *Tsc2* -/- mouse embryo fibroblasts (MEFs) were provided by H Onda (Brigham and Women's Hospital, Boston, USA;[5, 7]). Transfections were performed using lipofectAMINE and PLUS reagent, as recommended by the supplier (Invitrogen). Growth medium was replaced with DMEM without additives 24 h after transfection and after 24 h of serum starvation, cells were fixed in 3% paraformaldehyde for 10 min and permeabilised with 0.2% Triton X-100 for 5 min. Antibodies against epitope-tagged tuberin or hamartin, and S6 or phosphorylated S6, were incubated with the coverslips overnight at 4°C. Finally, the coverslips were incubated with fluorescein isothiocyanatecoupled and Texas Red isothiocyanate-coupled secondary antibodies against mouse or rabbit immunoglobulins, respectively (both obtained from DAKO) and studied using a LeicaDM RXA microscope. In each experiment, at least 50 transfected cells were analysed per coverslip.

Immunoblotting and immunoprecipitation

Cells (6cm dishes) were transfected using lipofectAMINE and PLUS reagent (Invitrogen). After 2 days, the cells were lysed in 0.5 ml TNE (50mM Tris-HCl, pH 8.0, 150mM NaCl, 50mM NaF, 0.5mM EDTA, 1% Triton X-100) for 10 min on ice and cleared by centrifugation at 10 000 g for 10 min at 4°C. Supernatants were analysed by SDS-PAGE followed by immunoblotting and enhanced chemiluminescence detection (Amersham).

For immunoprecipitations, 2 ml of the appropriate antibody was added to 400 ml of the supernatant and incubated on ice for 90 min before addition of 20 ml of a 50% suspension of Protein A-Sepharose beads. After gentle rotation for 90 min at 4°C, the beads were washed with TNE, resuspended in Laemmli loading buffer and analysed by immunoblotting.

To analyse the phosphorylation of exogenous S6K, human embryonal kidney 293 cells (3.5cm dishes) were cotransfected with 2B4, the S6K expression construct, and the *TSC2* and *TSC1* expression constructs. After 2 days, the cells were harvested directly in Laemelli loading buffer and analysed by immunoblotting.

In vitro assay of rheb GTPase activity

Recombinant GST-rheb was purified from Escherichia coli BL21 cells after induction with 0.5mM isopropyl-B-Dthiogalactopyranoside for 2 h at 28°C. The bacteria were lysed in 50mM Tris-HCl (pH 7.6), 1mM DTT, 150mM NaCl and 1% Triton X-100 containing 0.1 mg/ml lysozyme and, after sonication, GST-rheb (B10 mg) was purified on glutathione–agarose beads and incubated with 50 mCi [α32P]-GTP for 15 min at room temperature. After washing extensively with 20mM Tris-HCl (pH 7.6), 10mM MgCl2, 100mM NaCl, 0.1 mg/ml BSA, 4mM DTT to remove excess [α32P]-GTP, the GTP-bound GST-rheb was eluted in 100 ml PBS containing 30mM glutathione (pH 8.0).

Tuberin and hamartin were immunoprecipitated from 293 cells (6cm dishes). Immunoprecipitates were washed twice with 500 ml reaction buffer (20mM Tris-HCl (pH 7.6), 10mM $MgCl_2$, 100mM NaCl, 0.1 mg/ml BSA, 4mM DTT), resuspended in 8 ml reaction buffer and mixed gently at room temperature with 2 ml of the eluted GST-rheb. At appropriate time points, 5 ml of the reaction mixture supernatant was transferred to 2 ml of dissociation buffer (150mM EDTA, 5mM GTP, 5mM GDP, 50mM DTT, 2% SDS) and incubated at 68°C for 10 min prior to loading 1 ml on a PEI-cellulose thin-layer chromatography plate. Chromatography was performed in 0.75M KH_2PO_4 (pH 3.4) and the plates scanned on a Molecular Dynamics phosphorimager. Signal intensity was calculated using the ImageQuant software package.

RESULTS

We investigated the effects on tuberin function of 15 different amino-acid changes: R367Q, N525S, K599M, A607T, 609insS, R611Q, R611W, A614D, F615S, C696Y, V769E, L826M, R905Q, P1202H and G1556S. In previous work, we studied the effects of the N525S, K599M, R611Q, R611W, A614D, F615S, C696Y, V769E and R905Q substitutions on the tuberin–hamartin

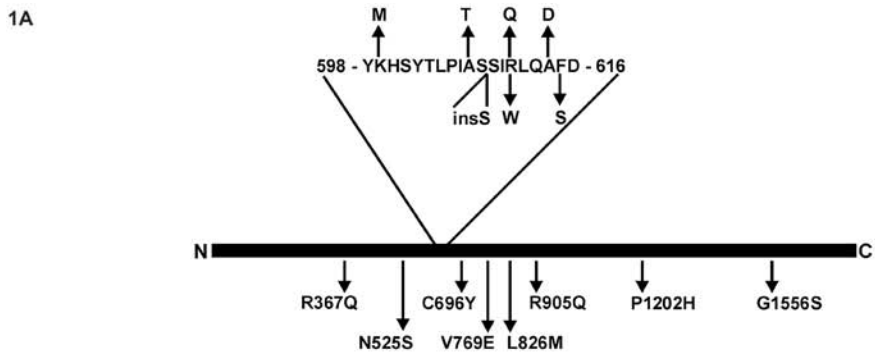
interaction, but not on the role of the proteins in the regulation of the mTOR signalling pathway[10]. The G1556S substitution has recently been described elsewhere[12]. The 609insS change (*TSC2* 1828insGCA) was identified in three affected members of a two generation TSC family and the P1202H substitution (*TSC2* 3623 C>A) was identified in a parent and child with TSC. The R367Q, A607T and L826M substitutions are nonpathogenic changes identified in unaffected relatives of TSC patients (Hodges et al[13]; O. Sancak, manuscript in preparation). The relative positions of the changes studied here are shown in Figure 1a.

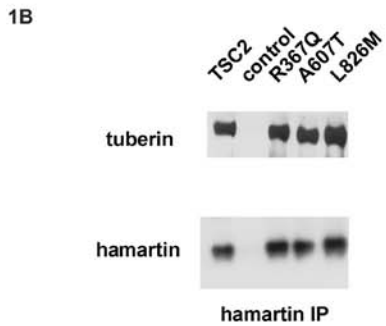
Effect of tuberin amino-acid changes on the tuberin–hamartin interaction

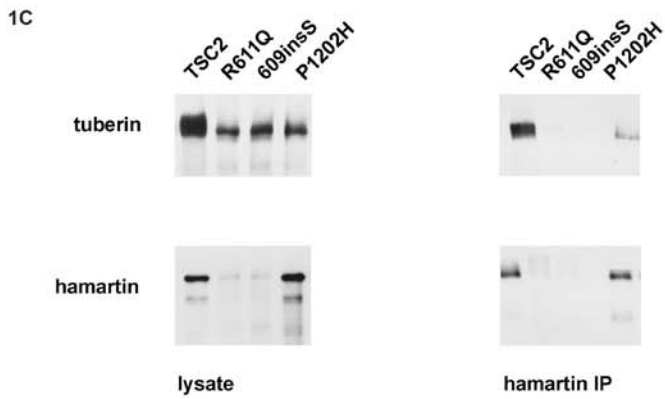
Previously, we demonstrated that the R611Q, R611W, A614D, F615S, C696Y and V769E substitutions disrupted the tuberin–hamartin interaction[10]. Hamartin expression was reduced in the presence of these tuberin variants compared to the wild-type protein and tuberin and hamartin could not be coimmunoprecipitated. The G1556S substitution also destabilised the tuberin–hamartin interaction, although expression of the G1556S tuberin variant did not reduce hamartin expression in cytosol[12].

The R367Q, A607T and L826M substitutions did not affect the tuberin–hamartin interaction. In each case, we were able to immunoprecipitate the tuberin–hamartin complex (Figure 1b). The 609insS tuberin variant had the same effect on the interaction as the R611Q, R611W, A614D, F615S, C696Y and V769E substitutions. As shown in Figure 1c, hamartin expression was reduced, and we were unable to coimmunoprecipitate hamartin and the 609insS tuberin variant. The P1202H substitution reduced, but did not prevent, coimmunoprecipitation of the tuberin–hamartin complex.

Figure 1 (next page)**:** Effects of single amino-acid changes on the tuberin–hamartin interaction. (a) Schematic diagram of tuberin indicating the relative positions of the 15 different amino-acid changes. The region of tuberin between amino acids 598 and 616 is shown in more detail. The positions of the K599M, A607T, 609insS, R611Q, R611W, A614D and F615S amino-acid changes are indicated. (b) Tuberin–hamartin complexes were immunoprecipitated with hamartin-specific antibodies from COS cells expressing exogenous hamartin, and tuberin (TSC2) or the R367Q, A607T or L826M variants. Hamartin and the tuberin variants were detected by immunoblotting. Mock-transfected cells were used as a control (control). (c) Hamartin, tuberin (TSC2) and the tuberin R611Q, 609insS and P1202H variants were overexpressed in COS cells. The expression of the proteins in the soluble fractions of the cell lysates was investigated by immunoblotting (lysate; left panel). Tuberin–hamartin complexes were immunoprecipitated with hamartin-specific antibodies (hamartin IP; right panel).


1A
M
T
Q
D
598 - YKHSYTLPIASSIRLQAFD - 616
insS
W
S
N
C
R367Q
C696Y
R905Q
P1202H
G1556S
N525S
V769E
L826M


1B
TSC2
control
R367Q
A607T
L826M
tuberin
hamartin
hamartin IP


1C
TSC2
R611Q
609insS
P1202H
tuberin
hamartin
lysate
TSC2
R611Q
609insS
P1202H
hamartin IP

Single amino-acid changes affect the phosphorylation of tuberin by PKB

We coexpressed the tuberin variants with active and inactive PKB isoforms. As shown in Figure 2a, expression of active PKB resulted in the appearance of low mobility isoforms of the N525S, K599M, R905Q, P1202H and G1556S tuberin variants. As shown in Figure 2b, only these low mobility tuberin isoforms were recognised by an antibody specific for the phosphorylated PKB target sites on tuberin (Phospho-(ser/thr) akt substrate antibody; Cell Signaling Technology[14]). We did not detect phosphorylation of the PKB target sites of the 609insS, R611Q, R611W, A614D, F615S, C696Y and V769E variants and we concluded that these amino-acid changes inhibit the phosphorylation of tuberin by PKB.

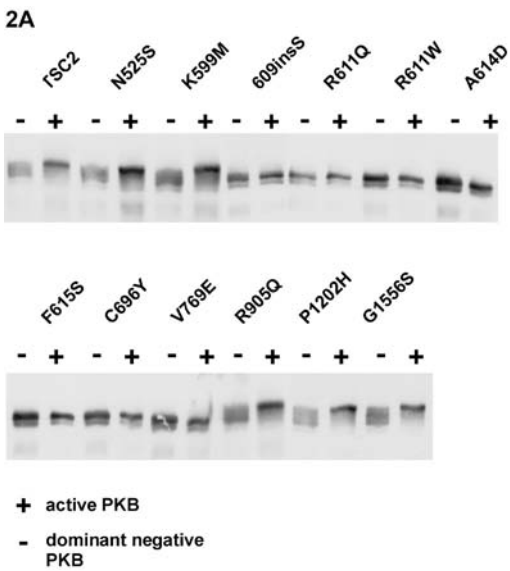
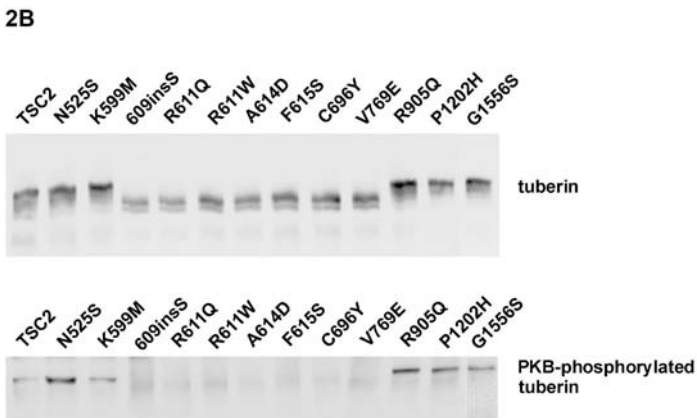


Figure 2: Single amino-acid changes affect the phosphorylation of tuberin by PKB. (a) The tuberin variants and either an active isoform of PKB (+) or a dominant-negative PKB isoform (-) were coexpressed in COS cells and in each case, the electrophoretic mobility of tuberin was assayed by immunoblotting. (b) The tuberin variants and an active PKB isoform were coexpressed in COS cells. The tuberin variants were immunoprecipitated and their electrophoretic mobilities compared by immunoblotting (upper panel). Phosphorylated PKB target sites on the immunoprecipitated tuberin variants were detected with the phospho-(ser/thr) akt substrate antibody (Cell Signaling Technology) (lower panel).



Effect of tuberin amino-acid changes on the tuberin–dependent inhibition of S6K phosphorylation

We overexpressed tuberin, hamartin and S6K in human embryonal kidney 293 cells, and analysed S6K phosphorylation by immunoblotting. As shown in Figure 3a, coexpression of tuberin and hamartin inhibited phosphorylation of the linker domain (T389) of the exogenous S6K. Next, we investigated the effect of expression of the tuberin variants on S6K T389 phosphorylation. As shown in Figure 3b, equal levels of the tuberin variants were expressed but S6K T389 phosphorylation was only reduced in cells overexpressing wild-type tuberin or the R367Q, N525S, K599M, A607T and L826M tuberin variants. Phosphorylation of S6K within the pseudosubstrate region (T421/S424) was unaffected by overexpression of hamartin and the tuberin variants.

The expression of hamartin was also dependent upon coexpression of the tuberin variants. A broad, diffuse band was detected when hamartin was expressed alone, or in the presence of the 609insS, R611Q, R611W, A614D, F615S, C696Y and V769E tuberin variants that do not form a complex with hamartin. This could be due to either increased ubiquitination[15] or phosphorylation[16] of hamartin when it is not bound to tuberin.

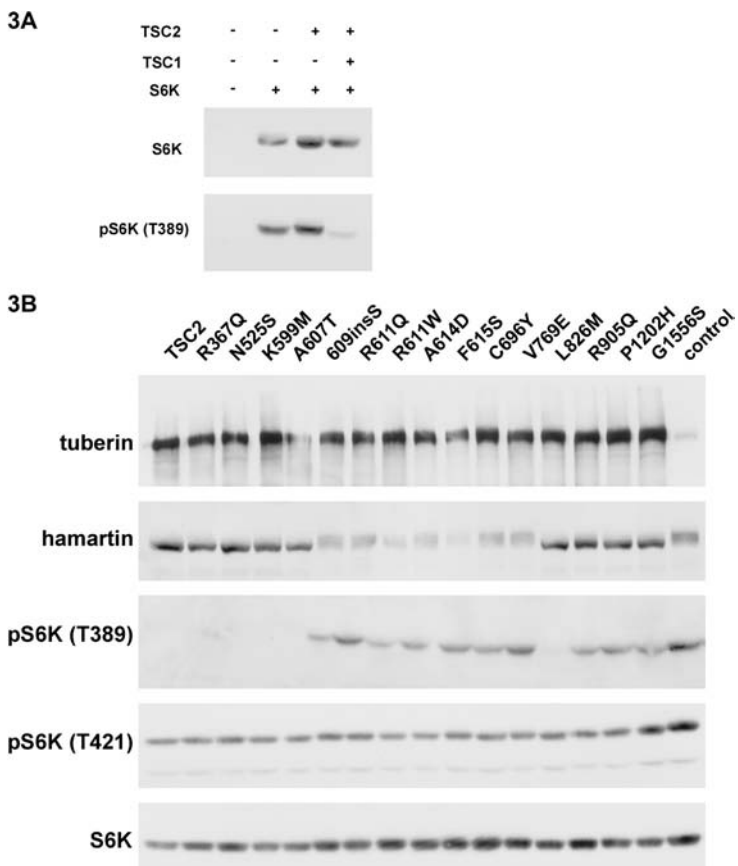


Figure 3:
Effect of tuberin and hamartin on the phosphorylation of S6K. (a) S6K was overexpressed in 293 cells alone, with tuberin, or with tuberin and hamartin. Phosphorylation of the linker domain (T389) of the exogenous S6K was assayed by immunoblotting. (b) S6K and hamartin were overexpressed in 293 cells together with wild-type tuberin and the 15 tuberin variants. Cells overexpressing hamartin and S6K only were included as a control (control). Expression of hamartin, S6K and the different tuberin variants, as well as the phosphorylation status of the overexpressed S6K was analysed by immunoblotting. Wild-type tuberin and the R367Q, N525S, K599M, A607T and L826M tuberin variants inhibited T389 phosphorylation of S6K but not T421/S424 phosphorylation. The 609insS, R611Q, R611W, A614D, F615S, C696Y, V769E, R905Q, P1202H and G1556S did not affect S6K phosphorylation.

Effect of tuberin amino-acid changes on the inhibition of S6 phosphorylation in Tsc2 -/- MEFs

After serum-starvation, the downstream effectors of mTOR (S6K, S6 and 4E-BP1) are phosphorylated in cells lacking either tuberin or hamartin, but not in wild-type cells[5-7]. We expressed tuberin and hamartin in *Tsc2* -/- and *Tsc1* -/- MEFs and assayed the phosphorylation of S6 in transfected, serum-starved cells by double-label immunofluorescent microscopy, as described previously by others[6, 17]. Transfected cells expressing either tuberin or hamartin, with a clear reduction in S6 phosphorylation, were counted 'blind' by two independent observers. Approximately 80% of the *Tsc2* -/- MEFs expressing exogenous tuberin and approximately 60% of the *Tsc1* -/- MEFs expressing exogenous hamartin had no detectable phosphorylated S6 (pS6), compared to less than 10% of control cells. We concluded that the inhibition of S6 phosphorylation in the *Tsc2* -/- and *Tsc1* -/- MEFs was due to the exogenous expression of tuberin and hamartin, respectively. Expression of both tuberin and hamartin together was more effective than expression of either protein alone; pS6 was detected in less than 20% of the transfected cells.

Next, we investigated whether coexpression of hamartin and the tuberin variants inhibited S6 phosphorylation in the *Tsc2* -/- MEFs, and whether expression of the tuberin variants alone was sufficient to inhibit S6 phosphorylation. As shown in Figure 4b, in cells expressing hamartin and the R367Q, N525S, K599M, A607T and L826M tuberin variants pS6 was not detected in approximately 90% of the transfected cells. There was no significant difference between wild-type tuberin and the R367Q, N525S, K599M, A607T and L826M variants in this assay (paired t-test $P>0.05$ in each case). In contrast, pS6 was detected in significantly more of the cells expressing the 609insS, R611Q, R611W, A614D, F615S, C696Y, V769E, R905Q, P1202H, and G1556S tuberin variants (paired t-test $P<0.05$ compared to wild-type tuberin in each case). These are the same amino-acid changes that prevented the tuberin–dependent inhibition of S6K T389 phosphorylation in the human 293 cells.

As shown in Figure 4c, expression of the tuberin variants alone (without hamartin) in the *Tsc2* -/- MEFs had slightly different effects on S6 phosphorylation. In approximately 70% of the *Tsc2* -/- MEFs expressing wildtype tuberin and the N525S and K599M tuberin variants, pS6 was not detectable, while in cells expressing the R611Q, R611W, A614D, C696Y, V769E, R905Q, P1202H and G1556S variants, pS6 was detected in <30% of the transfected cells (paired t-test $P<0.05$ in each case). Surprisingly however, in approximately half the cells expressing the tuberin 609insS and F615S variants, S6 phosphorylation was reduced. This was not significantly different to wild-type tuberin (paired t-test $P>0.05$ in each case) and suggested that the 609insS and F615S amino-acid changes did not inactivate the tuberin–dependent inhibition of S6 phosphorylation completely.

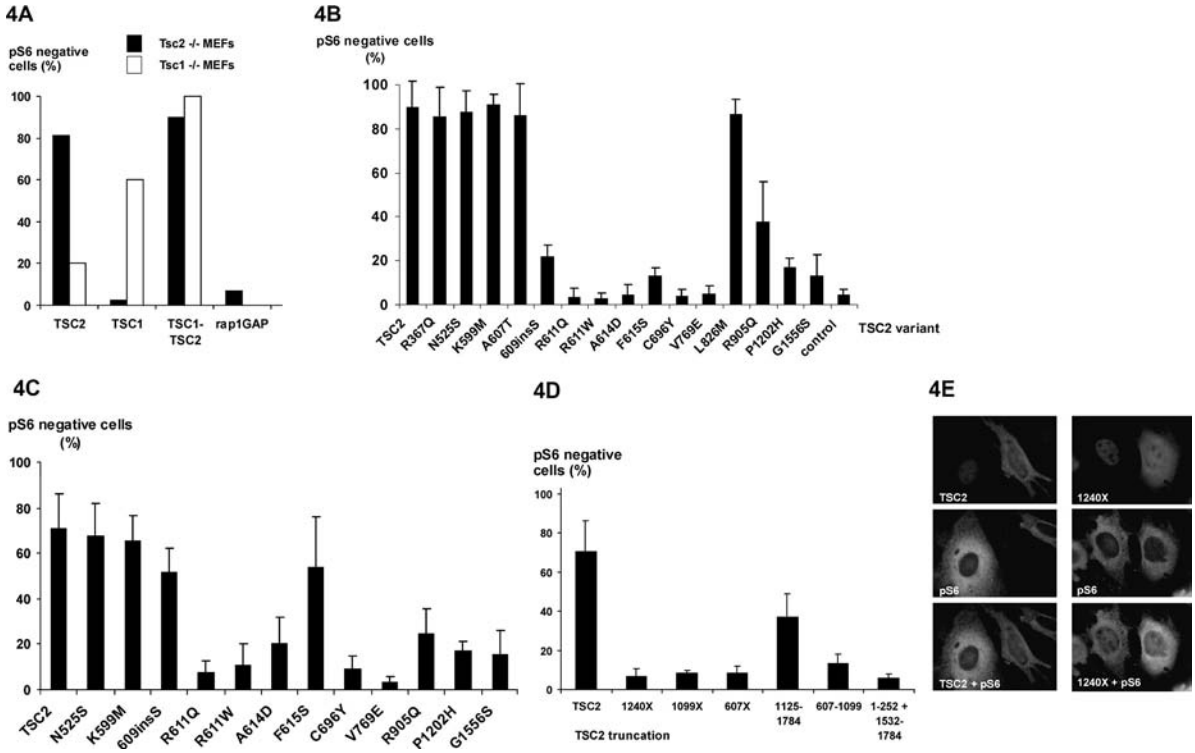


Figure 4: Tuberin and hamartin-dependent inhibition of S6 phosphorylation in *Tsc1* -/- and *Tsc2* -/- MEFs. (a) *Tsc1* -/- (open bars) and *Tsc2* -/- (solid bars) MEFs were transfected with expression constructs encoding myctagged hamartin (TSC1), polyhistidine-tagged tuberin (TSC2), both proteins (TSC1-TSC2) or V5-tagged rap1GAP. After serum starvation, double-label immunofluorescent microscopy was performed to assess S6 phosphorylation in the transfected cells. Phosphorylated S6 was detected using the phospho-S6 (S235/236) antibody from Cell Signaling Technology; the exogenous proteins were detected using antibodies against the different epitope tags. Cells expressing the exogenous proteins with detectable pS6 (no inhibition) or without detectable pS6 (inhibition) were counted. In each case, at least 50 cells were counted per transfection. The percentages of transfected cells expressing exogenous proteins and without detectable pS6 are indicated (pS6-negative cells). (b) *Tsc2* -/- MEFs were cotransfected with expression constructs encoding epitope-tagged hamartin and tuberin (TSC2), as before, and 15 epitope-tagged tuberin variants (R367Q, N525S, K599M, A607T, 609insS, R611Q, R611W, A614D, F615S, C696Y, V769E, L826M, R905Q, P1202H, and G1556S). After serum starvation, double-label immunofluorescent microscopy was performed to detect pS6 in the transfected cells, as described in (4a). The analysis was repeated five times. The mean values for the proportion of transfected MEFs without detectable pS6 are shown (pS6-negative cells). The proportion of MEFs overexpressing rap1GAP without detectable pS6 is also shown (control). (c) *Tsc2* -/- MEFs were transfected with expression constructs encoding epitope-tagged tuberin (TSC2) and 12 pathogenic epitope-tagged tuberin variants (N525S, K599M, 609insS, R611Q, R611W, A614D, F615S, C696Y, V769E, R905Q, P1202H, and G1556S). After serum starvation, double-label immunofluorescent microscopy was performed to detect pS6 in the transfected cells, as described in (4b). (d) *Tsc2* -/- MEFs were transfected with expression constructs encoding epitope-tagged tuberin (TSC2) and truncated tuberin proteins (1240X, 1099X, 607X, 1125-1784, 607-1099 and 1-252+1532-1784). After serum starvation, double-label immunofluoresent microscopy was performed to detect pS6 in the transfected cells, as described in (4b). (e) Representative examples of the inhibition of S6 phosphorylation in *Tsc2* -/- MEFs. Cells expressing epitope-tagged wild-type tuberin (TSC2) and a tuberin truncation protein (1240X) are shown in the top left and top right panels, respectively. Phosphorylated S6 (pS6) in the same cells is shown in the central panels and the bottom left and bottom right panels show the combined images. Phosphorylated S6 is not detectable in the cell expressing wild-type tuberin (left), unlike in the cell expressing the 1240X truncation protein (right) or the untransfected cells (both panels).

Effect of tuberin truncation on the inhibition of S6 phosphorylation in Tsc2 -/- MEFs

We investigated whether expression of a specific tuberin domain would be sufficient to inhibit S6 phosphorylation in *Tsc2* -/- MEFs. As shown in Figure 4d, pS6 was detected in 90% of the *Tsc2* -/- MEFs expressing the 607X (amino acids 1–607), 1099X (amino acids 1–1099) and 1240X (amino acids 1–1240) N-terminal domains of tuberin, a central domain of tuberin (amino acids 607–1099), or an in-frame deletion protein (amino acids 1–252 plus 1532–1784). In contrast, pS6 was detected in only 60% of the cells expressing the tuberin C-terminal domain (amino acids 1125–1784). This difference was significant (paired t-test $P<0.05$), indicating that expression of the tuberin C-terminal domain was sufficient to inhibit S6 phosphorylation in *Tsc2* -/- MEFs, although less effectively than full-length tuberin.

Effect of tuberin amino-acid changes on rheb GTPase activity in vitro

To investigate whether the tuberin amino-acid changes affected the rhebGAP activity of the tuberin–hamartin complex directly, we assayed the rhebGAP activity of the complex in vitro. Tuberin and myc epitope-tagged hamartin were coexpressed in 293 cells and tuberin–hamartin complexes immunoprecipitated with antibodies specific for the myc epitope, as shown in Figure 5a. As shown in Figure 5b, immunoprecipitates of the wild-type complex increased the rheb GTPase activity compared to immunoprecipitates from untransfected cells, or cells expressing exogenous hamartin only. Immunoprecipitates of hamartin with the tuberin N525S and K599M variants caused a similar increase in rheb GTPase activity. In contrast, immunoprecipitates from cells expressing the R611Q, R905Q, P1202H and G1556S variants did not increase the rheb GTPase activity above control levels.

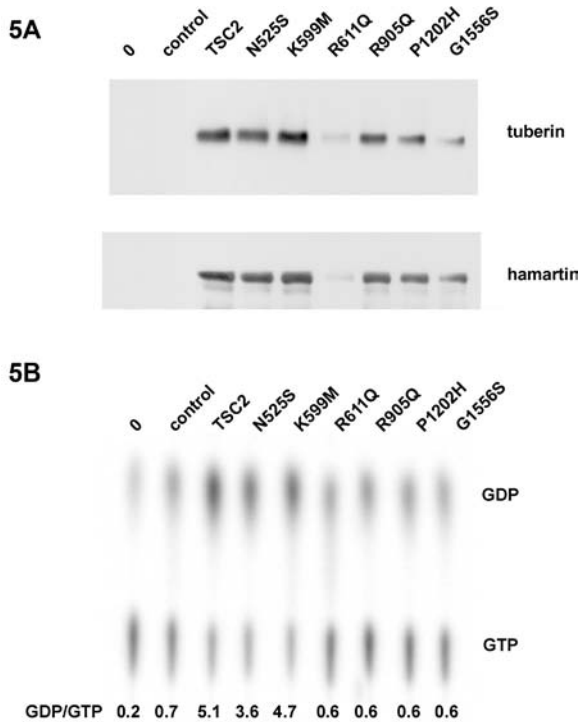


Figure 5: Effect of tuberin–hamartin complexes on in vitro rheb GTPase activity. Tuberin-hamartin complexes were immunoprecipitated from 293 cells with an antibody against the hamartin myc epitope tag and incubated with GST-rheb preloaded with [α-32P]GTP. GTP and GDP were resolved by thin-layer chromatography and the GTP:GDP ratio determined by phosphoimaging. After the GTPase assay, the immunoprecipitates were analysed by immunoblotting. Each tuberin variant was assayed at least 3 times. (a) Immunoblot of the immunoprecipitated tuberin-hamartin complexes used for the assay of rheb GTPase activity shown in (b). (b) Representative example of in vitro rheb GTPase activity measured in the presence of tuberin-hamartin complexes. The GDP:GTP ratios after incubation with the immunoprecipitates from untransfected cells (0), from cells overexpressing hamartin only (control), and from cells overexpressing hamartin and wild-type tuberin (TSC2), or the N525S, K599M, R611Q, P1202H and G1556S variants, are shown.

Some reports suggest that hamartin is necessary for the optimal GAP activity of tuberin[18-20]. However, other studies found that hamartin was not required for the stimulation of rheb GTPase activity by tuberin[21, 22]. To investigate whether the R367Q, N525S, K599M, 609insS, R611Q, R611W, A614D, F615S, C696Y, V769E, L826M, R905Q, P1202H and G1556S amino-acid changes affected the GAP activity of tuberin, the variants were overexpressed in 293 cells and immunoprecipitated with antibodies specific for tuberin. As shown in Figure 6a, equal amounts of the variants were immunoprecipitated. The wild-type tuberin immunoprecipitate caused a modest increase in rheb GTPase activity compared to the control immunoprecipitate of endogenous tuberin (Figure 6b). The R367Q, N525S, K599M, A607T, 609insS, F615S and L826M tuberin variants also increased the rheb GTPase activity. In contrast, the R611Q, R611W, A614D, C696Y, V769E, R905Q, P1202H and G1556S tuberin variants were unable to stimulate rheb GTPase activity above the control value (Figure 6c).

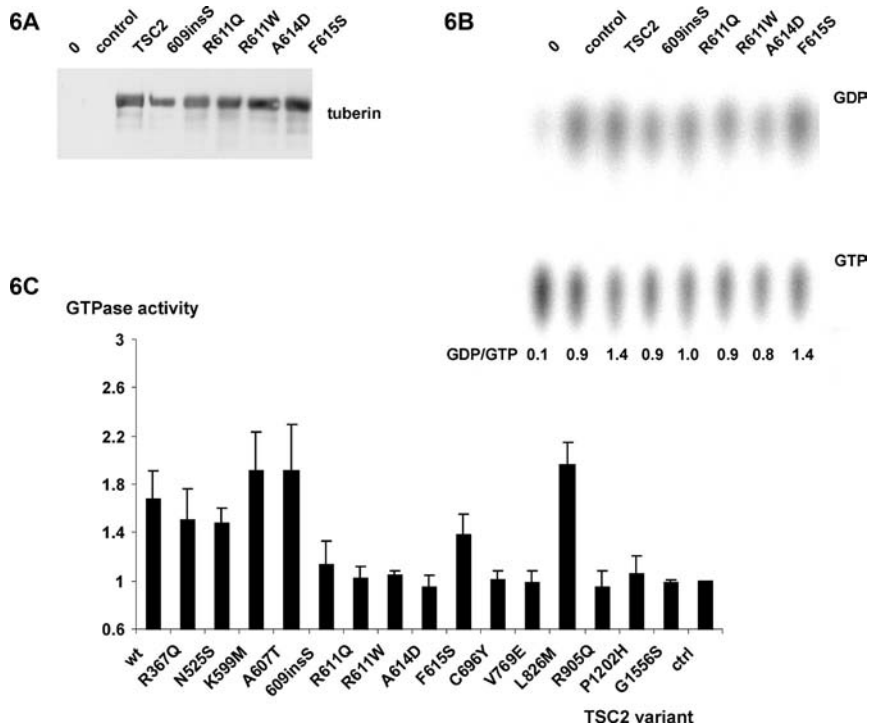


Figure 6: Effect of the tuberin variants on in vitro rheb GTPase activity. Immunoprecipitated tuberin variants were incubated with GST-rheb preloaded with [α-32P]GTP. GTP and GDP were resolved by thin-layer chromatography and the immunoprecipitates were analysed by immunoblotting. Each tuberin variant was assayed at least 3 times. (a) Immunoblot analysis of the immunoprecipitated tuberin variants used for the rheb GTPase activity assay shown in (b). (b) Representative example of an in vitro rheb GTPase assay in the presence of tuberin and the tuberin 609insS, R611Q, R611W, A614D and F615S variants. The GDP: GTP ratios after incubation with protein A beads only (0), from untransfected cells (control), and from cells overexpressing wild-type tuberin (TSC2), or the 609insS, R611Q, R611W, A614D and F615S variants, are shown. (c) GTPase activity of GST-rheb in the presence of the tuberin R367Q, N525S, K599M, A607T, 609insS, R611Q, R611W, A614D, F615S, C696Y, V769E, L826M, R905Q, P1202H and G1556S variants. The fold differences in the GTP:GDP ratio in the presence of exogenous wild-type tuberin and tuberin variants compared to endogenous tuberin (control) are shown. Each tuberin variant was tested at least three times.

DISCUSSION

We compared tuberin to 15 variants, each differing from the wild-type protein by a single amino-acid change. Three substitutions, R367Q, A607T and L826M, were identified in individuals without TSC and served as additional positive controls for tuberin activity. The K599M, R611Q, R611W, A614D, V769E and R905Q substitutions were originally identified as *de novo* changes in sporadic TSC patients[10]. The N525S, 609insS, P1202H and G1556S changes were identified in familial cases of TSC and cosegregated with the disease. The F615S and C696Y substitutions were also identified in TSC patients, although in these cases, DNA analysis of the parents could either not be performed, or could not establish whether the identified change was pathogenic[10].

We investigated the effects of the amino-acid changes on the tuberin–hamartin complex, tuberin phosphorylation, S6K phosphorylation and S6 phosphorylation. Finally, we assayed the effects of the changes on the in vitro stimulation of rheb GTPase activity by tuberin and the tuberin–hamartin complex. Our results are summarised in Table 1.

Tuberin variant	BLOSUM	Interaction with hamartin	Inhibition of pS6K (T389)	Inhibition of pS6	rheb GAP activity	Phosphorylated by PKB
Wild type	n/a	Yes	Yes	Yes	Yes	Yes
R367Q	1	Yes	Yes	Yes	Yes	Not done
N525S	1	Yes	Yes	Yes	Yes	Yes
K599M	-1	Yes	Yes	Yes	Yes	Yes
A607T	0	Yes	Yes	Yes	Yes	Not done
609insS	n/a	No	No	Yes	Weak	No
R611Q	1	No	No	No	No	No
R611W	-3	No	No	No	No	No
A614D	-2	No	No	No	No	No
F615S	-2	No	No	Yes	Weak	No
C696Y	-2	No	No	No	No	No
V769E	-2	No	No	No	No	No
L826M	2	Yes	Yes	Yes	Yes	Not done
R905Q	1	Yes	No	No	No	Yes
P1202H	-2	Weak	No	No	No	Yes
G1556S	0	Weak	No	No	No	Yes

Table 1: Comparison of the effects of amino-acid changes on tuberin function. Scores according to the BLOSUM 62 matrix25 are indicated

Results are detailed in the text and Figures 1,2,3,4,5,6.

Consistent with the identification of the R367Q, A607T and L826M substitutions in individuals without TSC, these changes did not adversely affect the tuberin–hamartin interaction, the stimulation of rheb GTPase activity or the inhibition of S6K and S6 phosphorylation. There was no evidence that the R367Q, A607T and L826M substitutions disrupted tuberin function and we concluded that they are nonpathogenic changes.

The R611Q, R611W, A614D, C696Y and V769E substitutions disrupted the tuberin–hamartin

interaction, and prevented the phosphorylation of tuberin by PKB, the inhibition of S6 and S6K phosphorylation, and the stimulation of rheb GTPase activity. All five substitutions inactivated tuberin completely in all our assays. We concluded that the R611Q, R611W, A614D, C696Y and V769E substitutions cause TSC because they result in major conformational changes to tuberin.

The 609insS and F615S amino-acid changes disrupted the tuberin–hamartin interaction, prevented phosphorylation of tuberin by PKB and prevented the inhibition of S6K phosphorylation. However, overexpression of the 609insS and F615S variants reduced S6 phosphorylation to almost the same extent as wild-type tuberin when overexpressed (in the absence of overexpressed hamartin) in *Tsc2* -/- MEFs, suggesting that the 609insS and F615S changes do not completely inactivate tuberin. The inability to form a stable complex with hamartin explains why the 609insS and F615S variants were less effective at inhibiting S6 phosphorylation in the cotransfection experiments (Figure 4b) than when overexpressed alone (Figure 4c). Interestingly, overexpression of the tuberin C-terminal domain in the *Tsc2* -/- MEFs, also reduced S6 phosphorylation in some cells, consistent with the suggestion that the tuberin C-terminal GAP domain is critical for tuberin activity[23, 24]. Our data suggest that the 609insS and F615S substitutions prevent tuberin activity in vivo because tuberin cannot be stabilised by the interaction with hamartin. The rheb GTPase activity measured in the presence of the 609insS and F615S variants was intermediate between the wild-type protein and the control values, suggesting that the 609insS and F615S variants retain some rhebGAP activity, consistent with the partial inhibition of S6 phosphorylation in the *Tsc2* -/- MEFs.

The R905Q, P1202H and G1556S substitutions did not prevent formation of the tuberin–hamartin complex, or the PKB-dependent phosphorylation of tuberin. However, the R905Q, P1202H and G1556S tuberin variants did not inhibit S6K phosphorylation in 293 cells, were less effective than wild-type tuberin at inhibiting S6 phosphorylation in the *Tsc2* -/- MEFs, and were unable to stimulate rheb GTPase activity. Our analysis indicates that although the R905Q, P1202H and G1556S substitutions do not prevent the formation of the tuberin–hamartin complex or tuberin phosphorylation, none of these variants increased the GTPase activity of rheb or inhibited S6 and S6K phosphorylation.

The N525S and K599M substitutions did not affect either the tuberin–hamartin interaction or tuberin phosphorylation[10]. Both variants inhibited the phosphorylation of S6K and S6, and increased the GTPase activity of rheb. We were unable to differentiate between wild-type tuberin and the N525S and K599M variants. Analysis of the respective nucleotide substitutions (*TSC2* 1292A4G and *TSC2* 1814A4T) with three different splice site prediction programs (http://www.genet.sickkids.on.ca/ali-cgi-bin/splicesitefinder; http://www.cbs.dtu.dk/cgi-bin/nph-webface?jobid¼netgene2 and http://www.fruitfly.org/cgi-bin/seq_tools/splice.pl) indicated that the mutations were unlikely to affect splicing of the *TSC2* transcript. As shown in Table 1, the N525S substitution had a neutral score in the BLOSUM 62 matrix[25] and was found to cosegregate with TSC in an affected family[26]. It is possible that this amino-acid change cosegregates with another (unidentified) *TSC2* mutation. Our analysis does not support the finding that the N525S

substitution is a pathogenic mutation. The K599M substitution is a *de novo* mutation[26] and has been shown to reduce the tuberin–dependent inhibition of phosphorylation of overexpressed 4E-BP1[20]. However, this effect is weak compared to the V769E tuberin variant[27]. Our analysis is consistent with the K599M substitution having a weak effect on tuberin function and it will be interesting to determine whether this mutation is associated with a mild TSC phenotype. It is possible that more sensitive assays will be required to distinguish some tuberin mutants from the wild-type protein. One way to do this would be to (stably) express endogenous levels of these variants in tuberin–deficient cells.

Our study indicates that nontruncating *TSC2* mutations can have distinct effects on tuberin function. It remains to be shown, however, whether these differences will correlate with differences in the phenotypes of TSC patients. In an earlier study, no evidence for a correlation between missense mutations to the tuberin GAP domain and a severe TSC phenotype was found[28]. Our study supports these findings. Amino-acid substitutions outside the GAP domain were sufficient to completely inactivate tuberin.

In summary, we have investigated the effects of pathogenic and nonpathogenic amino acid changes on tuberin function. We analysed the effect of the changes on the tuberin–hamartin interaction, on the phosphorylation of tuberin by PKB, and on the tuberin–dependent inhibition of S6 phosphorylation. Amino-acid changes to the central region of tuberin inactivated tuberin completely. Our data show that this central domain is necessary for formation of the tuberin–hamartin complex and indicates that missense mutations outside the tuberin GAP domain can completely inactivate the complex.

ACKNOWLEDGEMENTS

Financial support was provided by the Tuberous Sclerosis Alliance (USA), Noortman b.v., Maastricht, the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO). Dr H Onda (Brigham and Women's Hospital, USA) is thanked for supplying the *Tsc1* -/- and *Tsc2* -/- MEFs. Dr M van Slegtenhorst (Fox Chase Cancer Center, USA.) and Dr T Nobukini (Friedrich Miescher Institute, Switzerland) are thanked for supplying rheb and S6K expression constructs, and Dr AJJ Reuser is thanked for helpful discussions.

REFERENCES

1. van Slegtenhorst, M., et al., *Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34.* Science, 1997. **277**(5327): p. 805-8.
2. *Identification and characterization of the tuberous sclerosis gene on chromosome 16. The European Chromosome 16 Tuberous Sclerosis Consortium.* Cell, 1993. **75**(7): p. 1305-15.
3. Gomez, M., J.R. Sampson, and V.H. Whittemore, *Tuberous Sclerosis Complex*. 3rd ed. Developmental perspectives in psychiatry. 1991, New York and Oxford: Oxford University Press.
4. Kwiatkowski, D.J., *Tuberous sclerosis: from tubers to mTOR.* Ann Hum Genet, 2003. **67**(Pt 1): p. 87-96.
5. Zhang, H., et al., *Loss of Tsc1/Tsc2 activates mTOR and disrupts PI3K-Akt signaling through downregulation of PDGFR.* J Clin Invest, 2003. **112**(8): p. 1223-33.
6. Goncharova, E.A., et al., *Tuberin regulates p70 S6 kinase activation and ribosomal protein S6 phosphorylation. A role for the TSC2 tumor suppressor gene in pulmonary lymphangioleiomyomatosis (LAM).* J Biol Chem, 2002. **277**(34): p. 30958-67.
7. Kwiatkowski, D.J., et al., *A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells.* Hum Mol Genet, 2002. **11**(5): p. 525-34.
8. Manning, B.D. and L.C. Cantley, *Rheb fills a GAP between TSC and TOR*. Trends Biochem Sci, 2003. **28**(11): p. 573-6.
9. Li, Y., et al., *TSC2: filling the GAP in the mTOR signaling pathway.* Trends Biochem Sci, 2004. **29**(1): p. 32-8.
10. Nellist, M., et al., *TSC2 missense mutations inhibit tuberin phosphorylation and prevent formation of the tuberin-hamartin complex.* Hum Mol Genet, 2001. **10**(25): p. 2889-98.
11. van Slegtenhorst, M., et al., *Interaction between hamartin and tuberin, the TSC1 and TSC2 gene products.* Hum Mol Genet, 1998. **7**(6): p. 1053-7.
12. Mayer, K., et al., *Characterisation of a novel TSC2 missense mutation in the GAP related domain associated with minimal clinical manifestations of tuberous sclerosis.* J Med Genet, 2004. **41**(5): p. e64.
13. Hodges, A.K., et al., *Pathological mutations in TSC1 and TSC2 disrupt the interaction between hamartin and tuberin.* Hum Mol Genet, 2001. **10**(25): p. 2899-905.
14. Manning, B.D., et al., *Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway.* Mol Cell, 2002. **10**(1): p. 151-62.
15. Benvenuto, G., et al., *The tuberous sclerosis-1 (TSC1) gene product hamartin suppresses cell growth and augments the expression of the TSC2 product tuberin by inhibiting its ubiquitination.* Oncogene, 2000. **19**(54): p. 6306-16.
16. Astrinidis, A., et al., *Cell cycle-regulated phosphorylation of hamartin, the product of the tuberous sclerosis complex 1 gene, by cyclin-dependent kinase 1/cyclin B.* J Biol Chem, 2003. **278**(51): p. 51372-9.

17. Jaeschke, A., et al., *Tuberous sclerosis complex tumor suppressor-mediated S6 kinase inhibition by phosphatidylinositide-3-OH kinase is mTOR independent.* J Cell Biol, 2002. **159**(2): p. 217-24.
18. Garami, A., et al., *Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2.* Mol Cell, 2003. **11**(6): p. 1457-66.
19. Zhang, Y., et al., *Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins.* Nat Cell Biol, 2003. **5**(6): p. 578-81.
20. Tee, A.R., et al., *Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb.* Curr Biol, 2003. **13**(15): p. 1259-68.
21. Inoki, K., et al., *Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling.* Genes Dev, 2003. **17**(15): p. 1829-34.
22. Castro, A.F., et al., *Rheb binds tuberous sclerosis complex 2 (TSC2) and promotes S6 kinase activation in a rapamycin- and farnesylation-dependent manner.* J Biol Chem, 2003. **278**(35): p. 32493-6.
23. Jin, F., et al., *Suppression of tumorigenicity by the wild-type tuberous sclerosis 2 (Tsc2) gene and its C-terminal region.* Proc Natl Acad Sci U S A, 1996. **93**(17): p. 9154-9.
24. Momose, S., et al., *Identification of the coding sequences responsible for Tsc2-mediated tumor suppression using a transgenic rat system.* Hum Mol Genet, 2002. **11**(24): p. 2997-3006.
25. Henikoff, S. and J.G. Henikoff, *Amino acid substitution matrices from protein blocks.* Proc Natl Acad Sci U S A, 1992. **89**(22): p. 10915-9.
26. Niida, Y., et al., *Analysis of both TSC1 and TSC2 for germline mutations in 126 unrelated patients with tuberous sclerosis.* Hum Mutat, 1999. **14**(5): p. 412-22.
27. Tee, A.R., R. Anjum, and J. Blenis, *Inactivation of the tuberous sclerosis complex-1 and -2 gene products occurs by phosphoinositide 3-kinase/Akt-dependent and -independent phosphorylation of tuberin.* J Biol Chem, 2003. **278**(39): p. 37288-96.
28. Maheshwar, M.M., et al., *The GAP-related domain of tuberin, the product of the TSC2 gene, is a target for missense mutations in tuberous sclerosis.* Hum Mol Genet, 1997. **6**(11): p. 1991-6.

MILD PHENOTYPE IN TUBEROUS SCLEROSIS PATIENTS WITH CODON 905 MISSENSE MUTATIONS IN THE *TSC2* GENE

A. Jansen[1,2*], O. Sancak[3*], MD. D'Agostino[1,2*], A. Badhwar[1,2], P. Roberts[4], G. Gobbi[5], R. Wilkinson[6], D. Melanson[2,7], Tampieri[2,7], R. Koenekoop[8,9], M. Gans[9], A. Maat-Kievit[3], M. Goedbloed[3], A.M.W. van den Ouweland[3], M. Nellist[3], M. Pandolfo[10], M. McQueen[11], K. Sims[11], E. Thiele[12], F. Dubeau[2], F. Andermann[2,13], D.J. Kwiatkowski[4], D.J.J. Halley[3], E. Andermann[1,2,14]

1. Neurogenetics Unit, Montreal Neurological Hospital and Institute, Montreal, Quebec, Canada
2. Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec, Canada
3. Department of Clinical Genetics, Erasmus MC, Rotterdam, the Netherlands
4. Hematology Division, Brigham and Women's Hospital, Boston, Massachusetts
5. Department of Psychiatry, McGill University and Université de Montréal, Montreal, Quebec, Canada
6. Department of Dermatology, McGill University, Montreal, Quebec, Canada
7. Department of Radiology, McGill University, Montreal, Quebec, Canada
8. McGill Ocular Genetics Laboratory, Montreal, Quebec, Canada
9. Department of Ophthalmology, McGill University, Montreal, Quebec, Canada
10. Department of Neurology, Brussels Free University (ULB), Brussels, Belgium
11. Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts
12. Department of Pediatric Neurology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts
13. Department of Pediatrics, McGill University, Montreal, Quebec, Canada
14. Department of Human Genetics, McGill University, Montreal, Quebec, Canada

* These authors contributed equally to this work

Article submitted for publication

Corresponding author:
Eva Andermann M.D., Ph.D., F.C.C.M.G.
Neurogenetics Unit, Room 127
Montreal Neurological Hospital and Institute
3801 University Street
Montreal Quebec H3A 2B4 - Canada
Phone: 514 398 8529
Fax: 514 398 1276
Email: eva.andermann@mcgill.ca

Running Title: Codon 905 mutations in *TSC2*

ABSTRACT

OBJECTIVE: To report the clinical manifestations and functional aspects of codon 905 mutations in the *TSC2* tumor suppressor gene involved in tuberous sclerosis complex (TSC).

BACKGROUND: TSC is an autosomal dominant disorder characterized by hamartomatous growth in various organs, including the brain, and caused by mutations in the *TSC1* (9q34) or *TSC2* (16p13) genes. Overall, *TSC2* mutations have been associated with a more severe disease phenotype than *TSC1* mutations.

DESIGN/METHODS: We carried out a detailed study of the TSC phenotype in a large French-Canadian kindred (Family A). Linkage analysis of the *TSC* loci and mutation analysis of the *TSC2* gene were performed. In addition, clinical and molecular data on three families and six sporadic patients with mutations at the same codon were collected. Functional studies were performed on the codon 905 missense changes and related to the phenotypes.

RESULTS: A 2714G>A (R905Q) missense mutation in exon 23 of *TSC2* was identified in 25 individuals in Family A. The mild TSC phenotype in this family was characterized by absence of: visible cortical tubers, intractable epilepsy, disfiguring skin lesions, severe mental retardation or other major organ involvement. Diagnostic criteria were met in only a minority of family members, delaying diagnosis. Three other families with the same mutation were found to have a similar mild phenotype. Six unrelated TSC patients with a different mutation at the same codon (2713C>T or R905W) had a more severe phenotype. Both amino acid substitutions affected tuberin function and the results were consistent with the R905W substitution having a more severe effect on tuberin function than the R905Q substitution.

CONCLUSIONS: We have described ten new families with codon 905 missense changes in TSC2. In the R905Q families, the TSC phenotype was unusually mild, characterized mainly by depigmented skin lesions and by seizures that remitted spontaneously or that were easily controlled with anti-epileptic drugs. Functional studies showed both 905 codon mutations to be pathogenic without disrupting tuberin function completely. The finding of a more severe phenotype associated with the sporadic R905W mutation as compared to the familial R905Q mutation was consistent with the functional studies. Our findings support the observation that familial TSC is less severe than sporadic TSC, even when it is due to a TSC2 mutation. Genotype-phenotype correlations indicate that mild TSC phenotypes may be associated with specific TSC2 mutations.

Key words: tuberous sclerosis, genotype/phenotype correlations, mild phenotypes, functional studies

Abbreviations

AED	anti-epileptic drug
AML	angiomyolipoma
CS	confetti spots
CT	computed tomography
CTB	cortical tuber
DGGE	denaturing gradient gel electrophoresis
DHPLC	denaturing high-performance liquid chromatography
DNA	deoxyribonucleic acid
EEG	electroencephalogram
FAF	facial angiofibroma
FISH	fluorescent in-situ hybridization
FLAIR	fluid attenuated inversion recovery
FP	forehead plaque
GAP	GTPase activating protein
GDP	guanosine diphosphate
GFP	green fluorescent protein
GTP	guanosine triphosphate
HEK	human embryonic kidney
HMM	hypomelanotic macule
LAM	lymphangioleiomyomatosis
LOD	logarithm of the odds
MEF	mouse embryo fibroblast
MRI	magnetic resonance imaging
mTOR	mammalian target of rapamycin
rheb	ras homolog enriched in brain
SSCP	single stranded conformational polymorphism
SEGA	subependymal giant cell astrocytoma
SEN	subependymal nodule
SP	shagreen patch
TSC	tuberous sclerosis complex
UF	(sub)ungual fibromas

Introduction

Tuberous sclerosis complex (TSC) is a multisystem disorder characterized by the development of hamartias and hamartomas in various organs. Skin, brain, kidney, heart and eyes are most frequently involved, but virtually every organ may be affected [1]. The resulting phenotype is highly variable and can range from mild forms with skin lesions limited to hypomelanotic macules (HMM), normal intelligence, no or well controlled seizures and absence of other major organ involvement, to severe forms with disfiguring skin lesions, severe mental retardation, intractable epilepsy,

renal failure or lymphangioleiomyomatosis (LAM). Involvement of the brain is associated with some of the most severe clinical manifestations of TSC, including cognitive impairment, epilepsy and a variety of behavioral disorders such as autism and attention deficit with hyperactivity [2-5]. Life-threatening complications can be caused by respiratory insufficiency due to pulmonary lymphangioleiomyomatosis (LAM) or by subependymal giant cell astrocytoma (SEGA), renal cell carcinoma and renal angiomyolipoma (AML), depending on their size, location, and risk of spontaneous rupture or hemorrhage in the latter [6].

Tuberous sclerosis is estimated to occur in 1/6.000 - 1/10.000 live births [7, 8]. It is inherited as an autosomal dominant trait [9], and penetrance is considered to be complete [10]. Approximately 60%-70% of cases are sporadic, reflecting a high spontaneous mutation rate [7].

Linkage studies have shown locus heterogeneity for TSC [11-14], with disease determining genes on chromosomes 9q34 (*TSC1*: MIM *605284, 23 exons, gene product hamartin) [15] and 16p13 (*TSC2*: MIM *191092, 41 exons, gene product tuberin) [16]. *TSC1* mutations consist mainly of small deletions, insertions and nonsense mutations, as compared to *TSC2* mutations that also include missense mutations and large deletions and rearrangements [17]. *TSC1* mutations are less frequent than *TSC2* mutations, both in sporadic and familial cases [18]. Frequent loss of heterozygosity for alleles in 16p13.3 and rare loss in 9q34 have been observed in hamartomas from TSC patients, indicating that a second somatic mutation may be required to produce the TSC phenotype at the cellular level [19-21]. This observation is consistent with the *TSC1* and *TSC2* genes acting as growth suppressors. Hamartin and tuberin form a complex [22] that antagonizes mTOR (mammalian target of rapamycin)-mediated signalling [23].

Genotype/phenotype correlations in TSC have previously been addressed with partly conflicting results [18, 24, 25]. The phenotype is highly variable, both within and between families [7], and it is impossible to distinguish an individual patient with a *TSC1* mutation from a patient with a *TSC2* mutation on a clinical basis. Overall, *TSC2* mutations are associated with a more severe phenotype than *TSC1* mutations [18]. To date, the large majority of studies that have addressed genotype/phenotype correlations included only individuals that fulfilled the diagnostic criteria for TSC. This approach excludes individuals or relatives with possibly milder phenotypes due to either *TSC1* or *TSC2* mutations.

We report detailed phenotypic descriptions of a large French-Canadian kindred with a 2714G>A (R905Q) missense mutation in exon 23 of the *TSC2* gene, resulting in very mild phenotypes and little intrafamilial variability. Functional studies were consistent with the 2714G>A mutation being a mild mutation. In addition, we report three further families with the same 2714G>A mutation as well as six sporadic patients with a different mutation at codon 905 of *TSC2*. Genotype/phenotype correlations in TSC are discussed in the light of these findings.

Methods

Family A

Phenotypic characterization

Family A was ascertained at the Montreal Neurological Hospital and Institute. The proband presented with epilepsy and a detailed family history was obtained. The family included 220 individuals related to the proband. Two field trips were carried out to interview and examine the family members and collect blood samples on individuals who gave their informed consent according to a protocol approved by the Research Ethics Boards of the Montreal Neurological Hospital and Institute and the Centre Hospitalier de l'Université de Montréal. Medical records and brain imaging data were reviewed. Sixty individuals, 49 related to the proband, were examined. Forty-six had neurological examinations, 9 had neuropsychiatric and cognitive evaluations, 53 Woods lamp examinations, 26 ophthalmological examinations, 17 abdominal ultrasounds, 17 echocardiograms, 11 had electroencephalograms (EEG), 14 had both magnetic resonance imaging (MRI) and computed tomography (CT) scans, 4 had only CT and 2 had exclusively MRI. Of those who had MRI, fluid attenuated inversion recovery (FLAIR) sequences were obtained in 7. All imaging data were reviewed by DM. White matter lesions (WML) were classified either as TSC-related or as non-specific.

The revised diagnostic criteria for TSC [26] were applied to score 15 patients who had a full diagnostic work-up including brain imaging, dermatological, neurological and ophthalmological examinations, as well as abdominal and cardiac ultrasounds. The majority of family members were only partially examined and diagnostic criteria could not be fully scored. Partial examinations most often included a Woods lamp examination with in addition, either fundoscopy, brain imaging or renal ultrasound. Even if only one examination was missing, individuals were still reported as having a partial work-up. Fifteen individuals directly related to the proband were evaluated based on family history alone.

Linkage and mutation analyses

Linkage and mutation analyses were performed on 54 individuals. Genomic DNA (deoxyribonucleic acid) was isolated from venous blood leukocytes by phenol-chloroform extraction [27]. Linkage analysis was performed with the aim of determining whether the family linked to the *TSC1* or the *TSC2* locus. Microsatellite markers spanning the *TSC1* locus on chromosome 9q34 (D9S1847, D9S179, D9S1793) and *TSC2* locus on chromosome 16p13 (D16S3065, D16S3027, D16S3128) were utilized. Two-point lod (logarithmic odds) scores were calculated using the MLink program [28], Version 5.1. A single autosomal dominant model with 60% penetrance and 0.0001 disease allele frequency was used. Affected status was defined as the coexistence of skin lesions and epilepsy.

Mutation analysis was performed using denaturing high-performance liquid chromatography (DHPLC) screening for *TSC2* [29, 30] or a combination of denaturing gradient gel electrophoresis (DGGE), single stranded conformational polymorphism (SSCP), Southern analysis and fluorescent in-situ hybridization (FISH) [31]. Amino-acid residues were numbered according to the European Chromosome 16 Tuberous Sclerosis guidelines [16].

Additional patients and families

Family B and patients P1-P5 were ascertained at the Department of Clinical Genetics of the Erasmus Medical Centre of Rotterdam. The clinical data on these patients were obtained via a questionnaire on tuberous sclerosis.

Family C and patient P6 were seen by KS at the Department of Neurology, Massachusetts General Hospital (MGH), Harvard Medical School.

Family D was referred to the DNA diagnostic lab at the MGH.

Functional analysis

Full-length *TSC1* and *TSC2* expression constructs have been described previously [22]. Expression constructs encoding the tuberin R905Q and R905W variants were derived by site-directed mutagenesis using the Stratagene QuickChange kit. All constructs were sequenced completely. *TSC2* and *TSC2*-variants were also cloned into a pEGFP-C2 expression vector (Clontech).

Polyclonal rabbit antisera specific for human tuberin and hamartin have previously been described [22]. All other antibodies were purchased from Cell Signaling Technology.

Functional analysis of the tuberin R905Q and R905W mutants was performed in human embryonic kidney (HEK) 293 cells as described previously [32], except that S6 phosphorylation was determined in *Tsc2* -/- mouse embryo fibroblasts (MEF) expressing green fluorescent protein (GFP) -tagged tuberin variants.

Results

Family A (Table 1, Figure 1a)

Case illustrations

The proband, V-15, a 25-year-old man, started having seizures at age 8 years. Attacks consisted of brief episodes of altered consciousness with eye-lid flickering, often preceded by a sense of vibration in the head. Seizures were mainly diurnal, precipitated by sleep deprivation. Initial treatment with carbamazepine failed, but after introduction of phenytoin at age 12 years he had only occasional seizures. Because of gingival hyperplasia, he was switched back to carbamazepine at age 21. In the last 2 years, he has had a few small tonic attacks at night. He usually wakes up because of a strange sensation, after which he sits up and stiffens for 30 seconds. He has had one generalized tonic clonic seizure in the context of stress and non-compliance with his treatment. Early EEGs showed epileptiform activity over the left parieto-occipital head regions at age eight years. More recently, EEGs have been normal or showed mild generalized slowing. He has discrete left facial atrophy, mild slowing of rapid alternating movements of the fingers, and 2 HMM on the trunk. CT scan of the brain and eye exam were normal. He attended special classes in a regular school until grade 8, after which he started working in a sawmill.

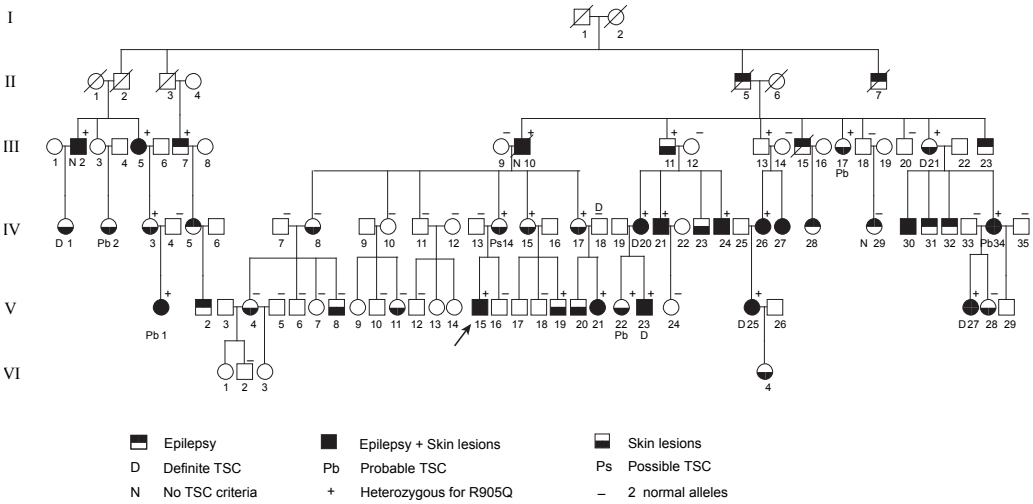


Figure 1a: Pedigree of Family A

His mother, IV-14, a 41-year-old woman, attended school to grade 7, and later worked as a nanny. She never had seizures. She has 12 HMM scattered from shoulders to ankles. Brain CT and MRI, including FLAIR sequences, abdominal and cardiac ultrasound, as well as eye examination were normal.

The maternal grandfather, III-10, died in his late seventies. He started having seizures at age 16 years, with an average of 2 attacks per week. Seizures were mainly nocturnal, could be preceded by a sensation of dizziness or vertigo, and were characterized by chewing and swallowing during sleep, sometimes with secondary generalization. Attacks were fully controlled with phenobarbital. Treatment was progressively diminished and he was seizure free without antiepileptic medication for 28 years. He left school at a young age, was aggressive and suffered from anxiety. He had a single HMM on the trunk. CT and MRI of the brain, cardiac and abdominal ultrasound, as well as eye exam were strictly normal.

Additional case reports are available in **Appendix.**

Molecular results

In Family A, linkage to *TSC1* was excluded by recombination and highly negative lod scores (data not shown). Linkage was demonstrated with markers spanning the *TSC2* locus on chromosome 16p13.3. The maximum lod score was 4.044 with marker D16S3027. Mutation analysis of the *TSC2* gene was performed. Twenty-five individuals were found to harbor a 2714 G>A substitution in exon 23 of *TSC2*, resulting in an arginine to glutamine missense change at codon 905 (R905Q).

Diagnostic work-up (Table 1)
Of the 25 mutation carriers, 12 had a complete diagnostic work-up. Five fulfilled criteria for definite TSC (III21, IV20, V23, V25, V27), 4 had probable TSC (III17, IV34, V1, V22), and 1 had possible TSC (IV14). The mutation was found in 2 individuals who did not fulfill any of the current criteria (III2 and III10), but who each had fewer than 3 HMM and epilepsy which remitted spontaneously. In 2 of 12 (IV14, V22), skin lesions consisting of >3 HMM and confetti spots (CS) were the only manifestation of the disease.

Work-up was incomplete in the remaining 13 mutation carriers, based on history in 1, skin exam in 8, skin-eye exam in 2, and skin-eye-brain exam in the remaining 2. Although not fully examined, 2 patients fulfilled criteria for probable TSC (IV3, IV24) and 2 had possible TSC (IV21, V19).

Twenty-nine individuals tested negative for the mutation. Of the 18 individuals related to the proband, five individuals had atypical skin lesions consisting of 1 to 3 hypopigmented spots (IV8, V4, V8, V11, V28), and one had seizures (IV29). Other investigations were normal. In the 11 individuals not related to the proband, no abnormalities were found.

Mutational status was not determined in 21 individuals related to the proband, on which information was available based on examination (9) or history (12). Patients IV-1 and IV-2 had a complete screening for TSC and fulfilled criteria for definite and probable TSC respectively. The remaining patients were only partly screened.

In summary, only 5 out of 12 mutation carriers who had a complete diagnostic work-up fulfilled the current diagnostic criteria for definite TSC, and the mutation was found in 2 individuals who did not fulfill any of the criteria. Mutational status was not determined in 5 relatives with possible or probable TSC based on skin lesions, and in 1 with definite TSC based on skin lesions and subependymal nodules (SEN). Epilepsy was present in 27 individuals, but is not part of the criteria for TSC. However, of 16 individuals with epilepsy who had molecular testing, 15 were positive for the mutation.

Clinical features of mutation carriers
Table 1 summarizes the clinical features in Family A. Mean age at last examination in the mutation carriers was 37.16 years (median 37; range 6-73).

1. Epilepsy
Fifteen mutation carriers (60%) had seizures. Detailed seizure history was available in 13. Age at seizure onset ranged from 15 months to 37 years, with a mean of 12 years. Nine (69%) became seizure free: 7 became seizure free without treatment and 2 were controlled with anti-epileptic drugs (AEDs). Age at seizure remission ranged from 4 to 49 years, with a mean age at remission of 26 years (median 34). There was partial seizure control with AEDs in the remaining 4.

Seizures were always focal in 4 (31%), focal with secondary generalization in 6 (46%), and generalized in 3 (23%). Infantile spasms were absent in all.

Family A					
Ped Num	Age (y) last exam	2714G>A	Criteria	Brain CT/MRI	Epilepsy
III:2	61	+	no	normal / normal	remitted
III:5	50	+	no-pt	n/a	remitted
III:7	58	+	no-pt	n/a	remitted
III:10	73	+	no	normal / normal	remitted
III:11	70	+	no-nt	n/a	never
III:13	67	+	no-nt	n/a	never
III:15	n/a	nt	no-nt	n/a	y-ns
III:17	42	+	probable	normal / WML	never
III:18	41	-	no-nt	n/a	never
III:20	n/a	-	no-pt	n/a	never
III:21	56	+	definite	normal / WML	never
III:23	n/a	nt	no-nt	n/a	y-ns
IV:1	28	nt	definite	2 SEN / n/a	never
IV:2	27	nt	probable	normal / normal	never
IV:3	28	+	probable-pt	n/a	never
IV:5	31	nt	no-nt	n/a	y-ns
IV:8	44	-	no-pt	n/a	never
IV:10	42	-	no-pt	n/a	never
IV:11	39	-	no-pt	n/a	never
IV:14	37	+	possible	normal / normal	never
IV:15	36	+	no-pt	n/a	never
IV:17	29	+	no-pt	n/a	never
IV:20	43	+	definite	normal / WML	remitted
IV:21	38	+	possible-pt	n/a	y-ns
IV:23	n/a	nt	possible-pt	n/a	never
IV:24	33	+	probable-pt	n/a	y-ns
IV:26	40	+	no-pt	normal / n/a	active
IV:27	36	nt	possible-pt	n/a	remitted
IV:28	n/a	nt	no-nt	n/a	y-ns
IV:29	37	-	no	normal / normal	y-ns
IV:30	33	nt	no-nt	n/a / normal	y-ns
IV:31	35	nt	no-nt	n/a	y-ns
IV:32	n/a	nt	no-nt	n/a	y-ns
IV:34	38	+	probable	n/a / normal	remitted
V:1	6	+	probable	normal / normal	active
V:2	7	nt	no-nt	n/a	y-ns
V:4	25	-	no-pt	n/a	never
V:6	23	-	no-pt	n/a	never
V:7	19	-	no-pt	n/a	never
V:8	16	-	no-pt	n/a	never
V:9	23	nt	no-nt	n/a	never
V:10	17	-	no-pt	n/a	never
V:11	16	-	possible-pt	n/a	never
V:12	17	-	no-pt	n/a	never
V:13	13	nt	no-nt	n/a	never
V:14	10	nt	no-nt	n/a	never
V:15	21	+	no-pt	normal / n/a	active
V:16	19	-	no-pt	n/a	never
V:17	18	nt	no-pt	n/a	never
V:18	15	-	no-pt	n/a	never
V:19	11	+	possible-pt	n/a	never
V:20	9	nt	possible-pt	n/a	never
V:21	8	+	no-pt	normal / n/a	remitted
V:22	22	+	probable	normal / normal	never
V:23	17	+	definite	normal / WML	remitted
V:24	9	-	no-pt	n/a	never
V:25	24	+	definite	SEGA / SEGA+WML	active
V:27	18	+	definite	R PV calc / 2 SEN	remitted
V:28	13	-	no-pt	n/a	never
V:29	n/a	nt	no-nt	n/a	never
VI:1	8	nt	no-pt	n/a	never
VI:2	5	-	no-pt	n/a	never
VI:3	2	nt	no-pt	n/a	never
VI:4	4	nt	possible-pt	normal / normal	never

Table 1: Clinical features in family A
age = age at last examination; AML = angiomyolipoma; criteria = diagnostic criteria for tuberous sclerosis (Roach et al. 1998); CS = confetti spot; HMM = hypomelanotic macule; LD = learning difficulties; MCI = mild cognitive impairment; n/a = not available; nt = not tested; oc = obligate carrier; Ped Num = pedigree number; pt = partially tested; R PV calc = right periventricular calcification; SEGA = subependymal giant cell astrocytoma; SEN = subependymal nodule; WML = white matter lesions; y = years; - no abnormality

Skin lesions	Kidney cyst/AML	Liver AML	Rhabdo-myoma	Retinal hamartoma	Cognitive function
2HMM	-/-	-	-	-	-
2HMM	n/a	n/a	n/a	-	-
-	n/a	n/a	n/a	-	-
1HMM	-/-	-	-	-	LD
HMM-nt	n/a	n/a	n/a	n/a	-
no-nt	n/a	n/a	n/a	n/a	LD
no-nt	n/a	n/a	n/a	n/a	n/a
6HMM	-/-	-	-	-	-
no-nt	n/a	n/a	n/a	n/a	n/a
-	n/a	n/a	n/a	n/a	n/a
>3HMM, >3CS	-/-	-	-	-	-
no-nt	n/a	n/a	n/a	n/a	n/a
1HMM, >3CS	-/-	-	-	+	-
>3HMM, >3CS	-/-	-	-	-	n/a
>3HMM, >3CS	n/a	n/a	n/a	n/a	LD
no-nt	n/a	n/a	n/a	n/a	LD
1HMM	n/a	n/a	n/a	n/a	n/a
-	n/a	n/a	n/a	n/a	n/a
-	n/a	n/a	n/a	n/a	n/a
12HMM	-/-	-	-	-	LD
2HMM	n/a	n/a	n/a	n/a	-
2HMM	n/a	n/a	n/a	n/a	LD
>3HMM, >3CS	-/-	-	-	-	-
3HMM, 2CS	n/a	n/a	n/a	n/a	LD
>3HMM	n/a	n/a	n/a	n/a	LD
>3HMM, >3CS	n/a	n/a	n/a	n/a	LD
>3HMM	n/a	n/a	n/a	n/a	MCI
2HMM, >3CS	n/a	n/a	n/a	n/a	LD
no-nt	n/a	n/a	n/a	n/a	n/a
-	-/-	-	-	-	MCI
yes-nt	n/a	n/a	n/a	n/a	n/a
yes-nt	n/a	n/a	n/a	n/a	n/a
no-nt	n/a	n/a	n/a	n/a	n/a
>3HMM, >3CS	-/-	-	-	-	LD
>3HMM, >3CS	-/-	-	-	-	MCI
no-nt	n/a	n/a	n/a	n/a	n/a
1HMM	n/a	n/a	n/a	n/a	n/a
-	n/a	n/a	n/a	-	LD
-	n/a	n/a	n/a	n/a	n/a
1HMM	n/a	n/a	n/a	n/a	n/a
no-nt	n/a	n/a	n/a	n/a	n/a
-	n/a	n/a	n/a	-	LD
3HMM	n/a	n/a	n/a	-	LD
-	n/a	n/a	n/a	-	LD
no-nt	n/a	n/a	n/a	n/a	n/a
no-nt	n/a	n/a	n/a	n/a	LD
2HMM	n/a	n/a	n/a	-	LD
-	n/a	n/a	n/a	-	LD
-	n/a	n/a	n/a	n/a	LD
-	n/a	n/a	n/a	n/a	LD
>3HMM	n/a	n/a	n/a	n/a	-
2HMM, 2CS	n/a	n/a	n/a	n/a	n/a
1HMM, >3CS	n/a	n/a	n/a	-	-
>3HMM, >3CS	-/-	-	-	-	-
>3HMM, >3CS	cyst/-	-	-	-	LD
-	n/a	n/a	n/a	n/a	n/a
>3HMM, >3CS	-/-	-	-	-	MCI
>3HMM, >3CS	-/AML	-	-	-	-
2HMM	-	-	n/a	n/a	n/a
no-nt	n/a	n/a	n/a	n/a	n/a
-	n/a	n/a	n/a	-	LD
-	n/a	n/a	n/a	n/a	n/a
-	n/a	n/a	n/a	n/a	n/a
2HMM, >3CS	n/a	n/a	-	-	n/a

Information on EEG was not available in most individuals from generations II and III. In the younger generations, EEGs showed epileptiform abnormalities in 6 and were normal in 5.

Of the 15 mutation carriers with seizures, 11 had brain imaging, which was normal in 7. Two had WML, 1 had SEGA and WML, and 1 had SEN.

2. Imaging

Brain imaging was available in 15 of 25 mutation carriers (60%). Three were evaluated by CT, 1 by MRI, and 11 had both CT and MRI, including FLAIR sequences in 7. Imaging abnormalities were present in 6 (40%). Four had TSC-related WML, which were exclusively frontal in 3 and more diffuse in 1. Patient V-25 had a single frontal WML and a left frontal SEGA in association with focal ventricular enlargement. Patient V-27 had 2 subependymal nodules. Four of 5 patients with definite TSC had WML.

Imaging was also performed in 4 individuals whose mutational status had not been determined (IV1, IV2, IV30, VI4). Two subependymal nodules were detected in patient IV-1; exams were normal in the other 3.

Cortical tubers were absent in all tested individuals.

Of the 6 mutation carriers with imaging abnormalities, 4 had epilepsy and all 4 fulfilled criteria for definite TSC. Two individuals with TSC-related WML (III7, III21) and 1 patient with 2 SEN (patient IV-1, mutational status unknown) did not have seizures.

Of the 9 mutation carriers with normal imaging, 7 had epilepsy.

3. Skin lesions

Skin lesions were present in 92% of mutation carriers and consisted only of hypomelanotic macules or confetti spots. Shagreen patches (SP), forehead plaques (FP), facial angiofibromas (FAF), and subungual fibromas (UF) were absent in all. Fifteen mutation carriers (60%) had 3 HMM or more, as is required for inclusion according to the latest diagnostic criteria. Confetti spots were present in 12 (48%). Apart from learning difficulties, skin lesions were the only abnormality found in 7 mutation carriers, but only 2 of these underwent complete screening. One to 3 hypomelanotic skin lesions were identified in 5 individuals who did not carry the mutation.

4. Renal and hepatic findings

Abdominal ultrasound was performed in 12 mutation carriers (age range 13 – 73) and was normal in 10. Patient V-22 had a single renal cyst; patient V-27 had a small renal AML. Abdominal ultrasounds were normal in 4 individuals related to the proband, whose mutational status was either undetermined (IV1, IV2) or negative (IV29, V28). The liver was free of lesions in all tested patients.

5. Cardiac findings
Cardiac ultrasound was performed in 12 mutation carriers and in 4 individuals related to the proband (mutational status unknown in 3, negative in 1). The investigations were performed during adolescence or in adulthood, except in patients V-1 and VI-4 who were examined at ages 6 and 4 respectively. No lesions were identified.

6. Ocular findings
Sixteen mutation carriers and 10 other family members (mutational status unknown in 4, negative in 6) had an ophthalmological examination including fundoscopy. Except for patient IV-1, who had a minor anomaly of the right optic nerve, which could represent a glial fold or a small hamartoma, all were within normal limits.

7. Learning difficulties and mild cognitive impairment
Learning difficulties defined as problems with normal schooling leading to failure of one or more grades or requiring special classes, were present in 10 mutation carriers (40%), as well as in 12 other family members (mutation status negative in 6). Three mutation carriers showed mild cognitive impairment, but all 3 were able to function in a sheltered environment and none lived in an institution.

In summary, despite incomplete work-up in 13 mutation carriers, skin lesions (HMM or CS) were present in 92%, epilepsy in 60%, learning difficulties or mild cognitive impairment in 52%, imaging abnormalities (WML, SEN or SEGA) in 24%, renal lesions (cyst, AML) in 8% and retinal abnormalities in 4%.

Families B to D and Patients 1 to 6

Clinical features of Families B, C, and D who carry the same mutation as Family A (2714G>A, R905Q, missense mutation in *TSC2* - exon 23), and of patients P1-P6 who have a different mutation in the same codon (2713C>T, R905W, missense mutation in *TSC2* - exon 23) are summarized in Table 2.

Family ID	Patient ID	F/S	Mutation	Criteria	Brain CT/MRI
Family B	I:1	F	2714G>A	definite	CTB
	II:1	F	2714G>A	definite	CTB
	II:2	F	2714G>A	possible-pt	normal
Family C	II:2	F	2714G>A	possible-pt	n/a
	III:2	F	2714G>A	definite	CTB
	III:5	F	2714G>A	possible-pt	n/a
	III:7	F	2714G>A	possible-pt	n/a
	IV:2	F	2714G>A	definite	n/a
	IV:3	F	2714G>A	possible-pt	n/a
	IV:5	F	2714G>A	n/a	n/a
	IV:7	F	2714G>A	definite	n/a
	IV:9	F	2714G>A	possible-pt	n/a
Family D	-	S-nc	2714G>A	definite	normal
P1	3925	S-c	2713C>T	definite	CTB, SEN
P2	2193	S-c	2713C>T	definite	CTB, SEN
P3	16365	S-nc	2713C>T	definite	CTB, SEN
P4	11489	S-c	2713C>T	definite	CTB, SEN
P5	21196	S-nc	2713C>T	definite	CTB, SEGA
P6	V	S-nc	2713C>T	definite	CTB, SEN

Table 2: Clinical Features in Other Families with Mutations of Codon 905. AML = angiomyolipoma; a-sx = asymptomatic; CI = cognitive impairment; CTB = cortical tubers; criteria = diagnostic criteria for tuberous sclerosis (Roach et al. 1998); FAF = facial angiofibroma; FP = forehead plaque; F = familial; HMM = hypomelanotic macule; (IS) = infantile spasms; (LG) = Lennox Gastaut syndrome; n/a = not available; ns = not specified; pt = partially tested; ret ham = retinal hamartoma; S-c = sporadic, parents clinically unaffected, do not carry mutation; SEGA = subependymal giant cell astrocytoma; SEN = subependymal nodule; S-nc = sporadic, parents clinically unaffected, no molecular testing; SP = shagreen patch; UF = peri/subungual fibroma

Epilepsy	Skin lesions	Kidney	LAM	Rhabdo-myoma	Eyes	CI
remitted	HMM, FAF	n/a	n/a	n/a	normal	no
never	HMM	n/a	n/a	n/a	normal	mild
remitted	HMM	n/a	n/a	n/a	normal	mild
never	HMM	n/a	n/a	n/a	n/a	no
remitted	HMM, UF	n/a	n/a	n/a	n/a	no
remitted	HMM	n/a	n/a	n/a	n/a	no
remitted	HMM	n/a	n/a	n/a	n/a	no
remitted	HMM, FAF, UF	n/a	n/a	+	normal	no
remitted	HMM	n/a	n/a	n/a	n/a	severe
n/a	n/a	n/a	n/a	n/a	n/a	n/a
never	HMM, SP	n/a	n/a	n/a	n/a	no
remitted	HMM	n/a	n/a	n/a	n/a	impaired-ns
active	HMM, SP	n/a	n/a	n/a	n/a	normal
never	HMM, FAF	n/a	n/a	n/a	normal	n/a
remitted	HMM, FAF, SP, FP	normal	n/a	-	ret ham	mild
active	HMM	normal	n/a	+	normal	moderate
active (LG)	HMM	normal	n/a	-	n/a	severe
never	HMM, FAF, FP	cysts	n/a	-	n/a	normal
remitted (IS)	HMM,FAF, SP, UF	AML	a-sx	n/a	n/a	mild

In Family B (Figure 1b), 2 of 3 mutation carriers had cortical tubers. Epilepsy was present in 2, with normal brain imaging in 1. Seizures were well controlled. Skin lesions were present in all and consisted of HMM, with additional FAF in 1. Two had learning difficulties. No retinal hamartomas were detected.

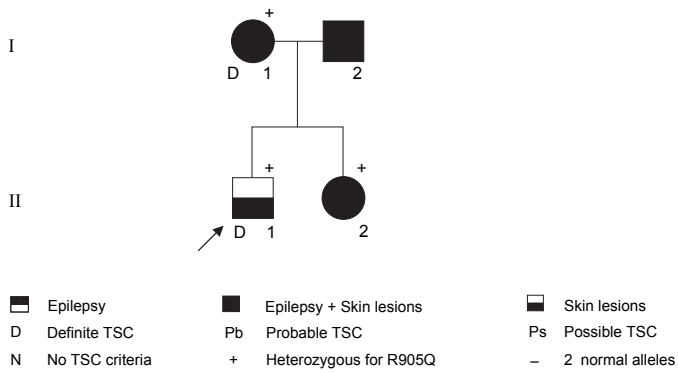


Figure 1b: Pedigree of Family B

In Family C (Figure 1c), cortical tubers were present in the single patient in whom brain imaging was performed. Six of 9 had epilepsy, which remitted spontaneously in all. HMM were found in all mutation carriers except in 1 (data not available), and 3 had additional skin lesions including FAF, UF and SP. One had cardiac rhabdomyoma. Two had cognitive impairment, which was severe in 1.

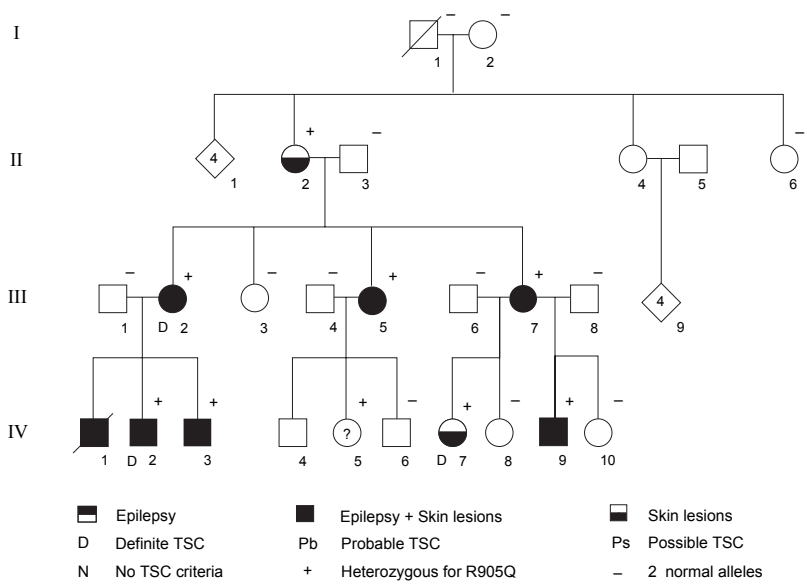


Figure 1c: Pedigree of Family C

In family D, the proband had normal brain imaging, epilepsy, HMM and SP. General intellectual functioning was normal. The paternal grandfather's half-sister had epilepsy but was never diagnosed to have TSC. The parents have not yet been tested for the mutation.

All 6 patients with the 2713C>T (R905W) mutation (P1-P6) had cortical tubers on brain imaging, with in addition SEN in 5 and SEGA in 1. Four of 6 had epilepsy, including 1 patient with Lennox-Gastaut syndrome and 1 with infantile spasms. Seizures remitted in 2. All patients had HMM with or without FAF, FP, SP, or UF. Additional organ involvement was present in 4 of 6. Four had cognitive impairment (1 not evaluated) ranging from learning difficulties to severe mental retardation. In the 3 R905W patients whose parents had mutation studies (P1, P2 and P4) the results were negative, confirming the *de novo* nature of the R905W mutation.

Functional studies

We compared the activity of wild type tuberin with the R905Q (2714G>A) and R905W (2713C>T) variants. We have previously studied the effects of the R905Q mutation on tuberin function [32, 33]. We now compared the effects of the R905Q and R905W substitutions on tuberin function.

Co-immunoprecipitation of tuberin and hamartin was not affected by the R905Q mutation, consistent with earlier results, but was reduced by the R905W substitution (Figure 2a). To investigate whether the R905Q and R905W substitutions affected the rhebGAP (ras homologue enriched in brain GTPase activating protein) activity of the tuberin-hamartin complex, the ability of immunoprecipitated wild type and mutant tuberin-hamartin complexes to stimulate the GTPase activity of rheb was tested *in vitro*. As shown in Figure 2b, the rheb-dependent conversion of guanosine triphosphate (GTP) to guanosine diphosphate (GDP) was reduced by both R905Q (GDP/GTP 0.6) and R905W (0.4) compared to wild type tuberin (1.9). The difference between R905Q and R905W was consistent in 3 independent experiments.

Earlier studies demonstrated that the tuberin R905Q mutant was less able to inhibit mTOR-mediated phosphorylation of p70 S6 kinase (S6K) and the S6K substrate, ribosomal protein S6 than wild type tuberin [32]. We compared the effect of expression of hamartin and either wild-type tuberin, or the R905Q and R905W mutants, on coexpressed S6K in HEK 293 cells. As shown in Figure 2c, the R905Q and R905W mutants inhibited S6K phosphorylation, but were less effective than wild-type tuberin. Consistent with the reduced inhibition of S6K phosphorylation by the tuberin R905Q and R905W mutants, expression of either mutant in *Tsc2* -/- MEFs was less able to inhibit phosphorylation of endogenous S6 than wild type tuberin (Figure 2d).

Table 3 contains a literature review of patients with either a 2714G>A or a 2713C>T missense mutation or with a mild TSC phenotype due to mutations other than 2714G>A/2713C>T, including Family E in our series.

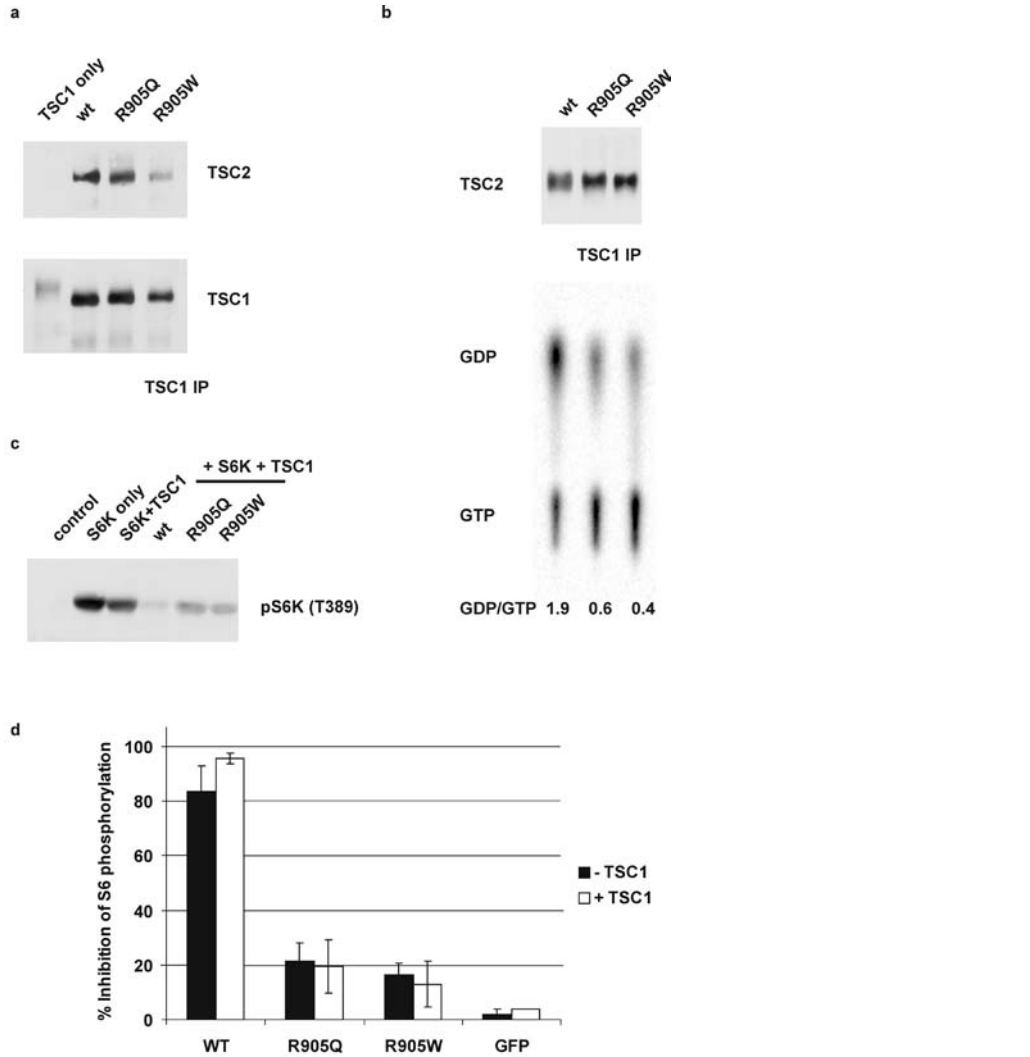


Figure 2: Effects of the R905Q and the R905W amino acid substitutions on tuberin function. (a) Tuberin-hamartin interaction: Tuberin-hamartin complexes were immunoprecipitated with hamartin-specific antibodies from 293 cells expressing exogenous hamartin (TSC1) and wild-type tuberin (wt), or the R905Q or R905W variants. Hamartin and the coimmunoprecipitated tuberin variants were detected by immunoblotting. (b) In vitro rheb GTPase activity of the tuberin-hamartin complex: Myc-tagged hamartin and wild-type tuberin (wt), the R905Q or the R905W tuberin variants were expressed in 293 cells. Tuberin-hamartin complexes were immunoprecipitated with an antibody against the myc epitope tag and incubated with GST-rheb preloaded with [α-^{32}P]GTP. GTP and GDP were resolved by thin-layer chromatography and detected by phosphoimaging. (c) Effect of tuberin and hamartin on linker domain (T389) phosphorylation of S6K: S6K and hamartin together with wild-type tuberin (wt) or the tuberin R905Q and R905W variants were overexpressed in 293 cells along with S6K and hamartin (S6K+TSC1), S6K only or empty vector (controls). (d) Tuberin and hamartin-dependent inhibition of S6 phosphorylation: GFP-tagged tuberin (wt) or the tuberin R905Q and R905W variants were overexpressed in *Tsc2* -/- MEFs either with or without hamartin. After serum starvation, S6 phosphorylation was determined in GFP positive cells by immunofluorescent microscopy. In each case, at least 50 cells were counted and the experiment was repeated six times. Cells expressing only GFP were used as control.

Reference	ID	F/S	Mutation	Age-Sex	Criteria	Brain CT/MRI	Epilepsy	Skin lesions	Kidney	Rhabdo-myoma	Eyes	CI	Dental Pits	LAM
Table 3A: Other families with codon 905 mutations														
Dabora et al. 2001	ONK921	S-nc	2714G>A	16y - n/a	definite	CTB ?	y	HMM, CS, FAF	n/a	n/a	n/a	no	n/a	n/a
Au et al. 1998	TS95-12	S-c	2713C>T	42mth - n/a	definite	SEN, CTB	yes - ns	HMM	normal	no	n/a	MR/DD	n/a	n/a
Jones et al. 1999	362	sib-gm	2713C>T	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Yamashita et al. 2000	1	S-c	2713C>T	n/a	definite	n/a	yes (IS)	HMM, FAF	n/a	n/a	n/a	yes (mod)	n/a	n/a
Yamamoto et al. 2002	8	S-nc	2713C>T	21y - f	definite	SEN, CTB	yes (complic)	HMM, FAF	AML	n/a	n/a	IQ 40	n/a	n/a
Yamamoto et al. 2002	7	S-nc	2713C>G	3y - m	definite	SEN, CTB	yes (West)	HMM	n/a	yes	n/a	n/a	n/a	n/a
Table 3B: Other families with mild TSC2 phenotypes														
Family E	II-3	F	4662G>A	n/a - f	definite	CTB	never	HMM,FAF, UF	AML	n/a	n/a	no	n/a	n/a
(Present study)	III-3	F	4662G>A	n/a - m	definite	CTB	never	HMM,FAF	n/a	n/a	n/a	no	n/a	n/a
	III-5	F	4662G>A	n/a - m	definite	CTB	never	HMM,FAF	n/a	n/a	n/a	no	n/a	n/a
	IV-3	F	4662G>A	n/a - f	definite	CTB	yes - ns	HMM,FAF	n/a	n/a	n/a	no	n/a	n/a
	IV-4	F	4662G>A	n/a - m	definite	CTB	never	HMM,FAF	n/a	n/a	n/a	no	n/a	n/a
	IV-7	F	4662G>A	n/a - f	definite	CTB, SEGA	never	HMM,FAF	n/a	n/a	n/a	no	n/a	n/a
	IV-8	F	4662G>A	n/a - f	definite	CTB	never	HMM,FAF	n/a	n/a	n/a	no	n/a	n/a
	IV-9	F	4662G>A	n/a - m	definite	CTB	never	HMM,FAF	n/a	n/a	n/a	no	n/a	n/a
	V-3	F	4662G>A	n/a - m	possible-pt	CTB	yes - ns	no	n/a	n/a	n/a	no	n/a	n/a
	V-4	F	4662G>A	n/a - m	possible-pt	CTB	never	no	n/a	n/a	n/a	no	n/a	n/a
Khare et al. 2001	F1	F	4508A>C		possible-pt	n/a	n/a	HMM	n/a	n/a	n/a	yes	n/a	n/a
Khare et al. 2001	F2	F	4508A>C		possible-pt	n/a	n/a	HMM	n/a	n/a	n/a	n/a	n/a	n/a
O'Connor et al. 2003	II-6	F	3106T>C	52y - m	definite	CM	yes - ns	6HMM	normal	no	normal	no	n/a	n/a
O'Connor et al. 2003	III-2	F	3106T>C	21y - m	no	normal	yes - ns	1HMM	normal	no	normal	no	n/a	n/a
O'Connor et al. 2003	III-5	F	3106T>C	20y - m	possible	CM	yes - ns	1HMM	normal	no	normal	no	n/a	n/a
O'Connor et al. 2003	III-6	F	3106T>C	9y - f	definite	CM	yes - ns	5HMM	normal	no	normal	no	n/a	n/a
O'Connor et al. 2003	III-1	F	3106T>C	12y - f	no	normal	yes - ns	no	normal	no	normal	no	n/a	n/a
O'Connor et al. 2003	III-4	F	3106T>C	22y - f	no	normal	never	no	normal	no	normal	no	n/a	n/a
O'Connor et al. 2003	II-2	F	3106T>C	32y - f	no	normal	never	no	normal	no	normal	no	n/a	n/a
Mayer et al. 2004	III-2	F	4684G>A	36y - f	definite	normal	never	HMM, SP<2mm	normal	n/a	normal	n/a	n/a	n/a
Mayer et al. 2004	III-3	F	4684G>A	35y - m	possible	normal	never	<3 HMM	AML	n/a	normal	n/a	n/a	n/a
Mayer et al. 2004	IV-1	F	4684G>A	11y - m	possible	normal	remitted	HMM	normal	n/a	normal	n/a	n/a	n/a
Mayer et al. 2004	IV-3	F	4684G>A	2mth - m	definite	normal	never	HMM	n/a	yes	n/a	no	n/a	n/a

Table 3: Literature Review. AML = angiomyolipoma; CI = cognitive impairment; CM = cortical malformation; (complic) = complicated; criteria = diagnostic criteria for tuberous sclerosis (Roach et al. 1998); CS = confetti spots; CTB = cortical tuber; f = female; FAF = facial angiofibroma; F/S = familial/sporadic; gm = germline mosaicism; HMM = hypomelanotic macule; (IS) = infantile spasms; m = male; MR/DD = mental retardation/developmental delay; mod = moderate; mth = months; N/A = not available; ns = not specified; rhabdo = cardiac rhabdomyoma; S-c = sporadic, parents clinically unaffected, do not carry mutation; SEN = subependymal nodule; S-nc = sporadic, parents clinically unaffected, no molecular testing; SP = shagreen patch; UF = peri/subungual fibroma; (West) = West syndrome; y = years

Discussion

Mild phenotypes in TSC can be characterized by absence of: disfiguring skin lesions, intractable epilepsy, mental retardation and severe organ involvement. It is generally believed that *TSC2* mutations are associated with a more severe phenotype than *TSC1* mutations [18, 34]. However, patients with *TSC2* mutations may be mildly affected and may not even fulfill the current diagnostic criteria. In this paper, we present 10 new families or index patients with mild expression of TSC and mutations in the *TSC2* gene.

Previous work has addressed the phenotypic differences between familial and sporadic TSC, and between patients with *TSC1* and *TSC2* mutations, as well as the possible relationship between the phenotype and the type of mutation, and intra- and interfamilial variability.

In a study of 123 TSC families, Au et al. found less severe neurological findings as compared to studies focused on sporadic patients [24]. Sancak et al. reported a less severe phenotype for both TSC1 mutations (familial and sporadic) and familial TSC2 mutations, as compared to *de novo* mutations in TSC2 [31]. An earlier study [18] found only a lower frequency of seizures ($P = 0.055$) and HMM ($P = 0.02$) in familial as compared to sporadic TSC patients, and these findings could not be reproduced when considering (familial and sporadic) cases with *TSC2* mutations exclusively. A less severe phenotype in familial versus sporadic TSC cases does seem likely however, since this is in keeping with a higher reproductive fitness in mildly affected individuals [35]. The study by Dabora et al. compared the clinical features in sporadic patients with those seen in index members of familial cases, and patients in both groups by definition had to fulfill the diagnostic criteria for TSC [26]. This study [18] did not examine or include affected family members, excluding individuals with a possibly milder phenotype who did not fulfill the diagnostic criteria. The different study designs can account for the differing results among the three studies.

The phenotypes associated with *TSC1* mutations were found to be less severe than the phenotypes associated with *TSC2* mutations, considering either the frequency of mental disability [34, 36, 37] or the TSC phenotype as a whole [18, 31, 38], in 6 studies with a total of 134 patients with *TSC1* mutations and 519 patients with *TSC2* mutations. However, no significant phenotypic differences were found in three smaller studies (41 patients with a *TSC1* mutation and 64 with a TSC2 mutation) [39-41]. Most likely, this discrepancy results from ascertainment bias (including the age-dependent nature of many TSC manifestations) or from the small number of patients included in some of the studies. Since all patients in the above-mentioned studies fulfilled the criteria for definite TSC; individuals with milder phenotypes were excluded.

TSC1 mutations are underrepresented in both sporadic and familial TSC patients [34, 37, 40]. In familial TSC, early linkage studies [42] reported a fifty-fifty distribution between the *TSC1* and *TSC2* loci, although more recent studies have shown underrepresentation of *TSC1* mutations in familial TSC as well [18, 31].

Several hypotheses have been put forward to explain the milder phenotype in patients with

mutations in *TSC1*. The underrepresentation of *TSC1* mutations is likely due to the higher germline mutation rate at the *TSC2* locus than at the *TSC1* locus [34]. If the phenotype is in part related to the frequency of the second hit, the milder phenotype in patients with mutations in *TSC1* can be explained by the lower somatic mutation rate at the *TSC1* locus than at the *TSC2* locus [18, 31]. In addition, tuberin may have functions that are not shared by hamartin [34]. Both the lower mutation rate and the milder phenotype in TSC1 disease may result in higher reproductive fitness and consequently a higher proportion of familial cases with a *TSC1* mutation.

No correlation was identified between the type of mutation and the phenotype for either *TSC1* or *TSC2*, except for increased severity of renal symptoms related to large deletions at the *TSC2* locus which disrupt the neighbouring gene *PKD1* as well [18, 25, 31, 34, 36, 37, 39, 40, 43-46]. In addition, there is extensive phenotypic variability for identical mutations, as illustrated by the high inter- and intrafamilial variability [18, 40, 44, 45], as well as by the discordant phenotypes described in monozygotic twins [47, 48]. It therefore seems likely that the nature of the second hit [21], somatic mosaicism [49], modifying genes, and environmental factors may have a major impact on the phenotypic expression of the germline mutation.

The proband in Family A presented with epilepsy and the family was first labeled as having familial partial epilepsy. The presence of skin lesions then led to the diagnosis of TSC, but since the epilepsy phenotype was very mild, the involvement of a third TSC locus was considered. The mild TSC phenotype in this family was characterized by the complete absence of disfiguring skin lesions in all affected family members. Fewer than 2/3 had epilepsy, which in the majority remitted spontaneously or was easily controlled by AEDs, and infantile spasms were absent. Cortical tubers were absent in all family members studied. It is not excluded however, that subtle lesions might be present in these patients and could be demonstrated using 3 Tesla MRI with surface coils or other imaging techniques, including post-processing techniques such as curvilinear reformatting or diffusion tensor analysis. A single AML and an isolated renal cyst were the only renal abnormalities detected. Since 80% of children with TSC have kidney lesions by the age of 10.5 years [50], it is unlikely that additional patients in the studied sample (mean age at examination 37 years) will develop TSC related renal abnormalities. Only 3 had mild cognitive impairment, and none were mentally retarded (Full Scale IQ level <70).

The presence of a mild phenotype in a pedigree of this size challenges the notion of extensive clinical variation in TSC. Possibly, this reputation is derived, at least in part, from studying severely affected index patients with mildly affected parent pairs without further familial context. Mild intrafamilial variability was however documented in Family A, ranging from phenotypes limited to isolated HMM, to phenotypes including SEGA, epilepsy, mild cognitive impairment and renal AML.

Patient III-13 was apparently non-penetrant for the phenotype, but since he did not have a complete work-up for TSC, no final conclusion can be drawn in this case.

The mutation was absent in patient IV-29 who had epilepsy and mild cognitive impairment, as well

as in patients IV-8, V-4, V-8, V-11, and V-28 who had 1 to 3 depigmented skin lesions, though not of classical ash-leaf configuration. These findings are not surprising considering the relatively high prevalence of both epilepsy and depigmented skin lesions in the general population, and their occurrence in Family A might well be related to chance.

On the other hand, only a minority of mutation carriers fulfilled diagnostic criteria for definite TSC (Table 1, and Figure 1a). However, when features such as seizures or having a family member with TSC were taken into account as well, a diagnosis of presumptive TSC could have been made in all mutation carriers. If mutation analysis had been restricted to the individuals who fulfilled the criteria for definite TSC, many family members would still be uncertain about the possibility of developing the disease or passing it on to their offspring. They would also have to face regular clinical check-ups with the associated stress and extra costs. Screening family members of individuals with a known *TSC* mutation regardless of the criteria will reduce the uncertainty in these families and will also save the costs of yearly check-ups. This test should be offered in the context of genetic counseling, and appropriate follow-up should be provided. Changing the diagnostic criteria in order to facilitate testing of family members at risk does not seem advisable. The criteria have been developed in order to make a correct clinical diagnosis and not to select individuals for molecular testing. Including a first degree relative with a molecular diagnosis of TSC as a diagnostic criterion will result in an increase of false positive TSC patients. In Family A, this would be the case for patient IV-29 who had epilepsy and mild cognitive impairment, as well as for patients IV-8, V-4, V-8, V-11, and V-28 who had 1 to 3 depigmented skin lesions.

We studied 3 additional families with the R905Q mutation and six patients with the R905W mutation (Table 2, Figures 1b and 1c).

With respect to the R905Q families (Families B, C, and D), the overall phenotype was mild, consistent with the phenotype described in Family A. Seizures had been present in 2/3 of patients in Families B, C, and D, which is similar to Family A (60%). Cortical tubers were present in 2 of the 3 families and disfiguring skin lesions were present in a minority of patients, in contrast to Family A. In the literature, 1 more patient with an R905Q mutation and a relatively mild phenotype (cognitively normal, no SEN or SEGA, epilepsy phenotype not available) has been mentioned. The patient was reported as sporadic, but mutational status was not determined in the parents [18] (Table 3A).

The sporadic R905W patients (Patients 1-6) had more severe phenotypes. All patients had cortical tubers (CTB), and SEN or SEGA. Seizures were present in 4 out of 6 as compared to approximately 60% of the R905Q cases, and were more difficult to control. HMM were more frequently associated with more severe skin lesions including FAF, SP, FP and UF. Cognitive impairment was present in 4 of 5 evaluated patients and ranged from mild to severe. Four of 5 patients had additional TSC features.

In the literature, 4 more patients with R905W [34, 38, 39, 43] and one patient with R905G [38] have been described (Table 3A). Despite incomplete information, phenotypes closely resemble

the ones described in P1-6, and appear more severe than the phenotypes of the familial cases with R905Q.

The identification of R905Q and R905W as recurrent mutations in 4 families and 11 sporadic cases, as well as the cross-species conservation of arginine at codon 905 (Figure 3), illustrate the importance of this amino acid for the function of tuberin. In order to better understand the relationship between the 2714G>A (R905Q) and 2713C>T (R905W) mutations and the TSC phenotype, the effects of the R905Q and R905W substitutions on tuberin function were studied. Unlike some other pathogenic amino acid substitutions, R905Q and R905W did not prevent formation of the tuberin-hamartin complex [33]. However, the R905W mutation destabilized the complex as compared to both wild type tuberin and R905Q. In addition, although the *in vitro* rhebGAP activity of the tuberin-hamartin complex was inhibited by both the R905Q and R905W changes, the R905Q mutant protein had slightly more rhebGAP activity than the R905W mutant. In two separate assays, both substitutions reduced the tuberin-dependent inhibition of signal transduction through mTOR. In conclusion, both changes clearly affected tuberin function confirming their pathogenicity. However, neither mutation seemed to inhibit tuberin function completely. The less severe effect of R905Q in our *in vitro* assays of tuberin-hamartin complex formation and rhebGAP activity is consistent with the association between the R905Q substitution and mild TSC phenotypes, enabling its familial presentation. These studies would suggest that different missense mutations in the same codon resulting in a different amino acid change can modify the severity of TSC.

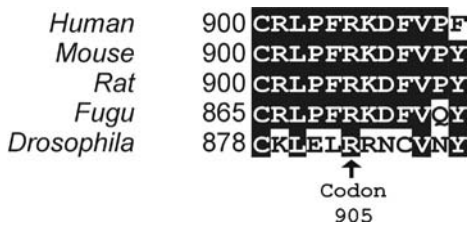


Figure 3: Evolutionary conservation of TSC2 exon 23. Amino acids that are conserved in 3 or more species are shown with a black background and white letters. Codon R905 is indicated with an arrow.

Although, in general, *TSC2* mutations are associated with more severe disease than *TSC1* mutations, mild phenotypes in patients with *TSC2* mutations do exist and are not limited to codon 905 changes (Table 3B). Family E (Figure 1d) demonstrates an exceptionally mild TSC2 phenotype in ten individuals, caused by a 4662G>A splice site mutation in exon 35 of the *TSC2* gene. All patients were cognitively normal, and seizures were present in only 20%. However, all patients had cortical tubers and eight had FAF, as opposed to the R905Q families. This would indicate that, in contrast to some previous studies, the type and location of the mutation can influence the phenotype. In Family E, 8 out of 10 patients fulfilled the current diagnostic criteria for TSC because of the presence of HMM, FAF and CTB, although very few had epilepsy and all were cognitively normal. This is in contrast to Family A, in which 60% had epilepsy, 40% had learning difficulties, but only a few fulfilled the criteria.

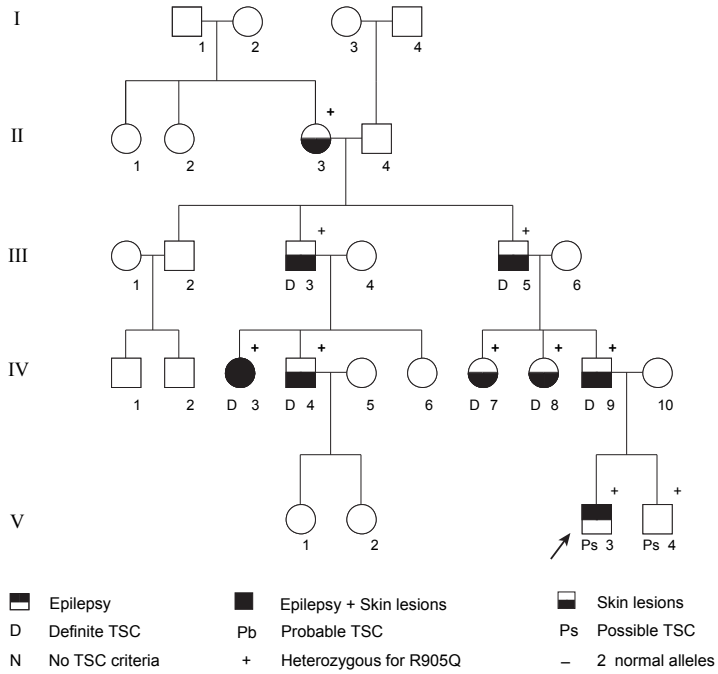


Figure 1d: Pedigree of Family E

Khare et al. described two families with a 4508A>C (1503Q>P) missense mutation in exon 34 of the TSC2 gene and, as far as details were available, a phenotype consisting mainly of HMM with in addition psychiatric features in 1 of the families [51]. O'Connor et al. reported a three generation family in which seven individuals had a 3106T>C (1036S>P) missense mutation in exon 26 of the *TSC2* gene. All were cognitively normal, skin lesions were limited to HMM and seizures were controlled either by AED or surgery [52]. Mayer et al. identified a family in which a very mild TSC phenotype was segregating with a 4684G>A (1556G>S) missense mutation in exon 36 of *TSC2*. Functional studies supported the pathogenic character of the substitution [53].

In conclusion, we report a large TSC family in which epilepsy was the only burden of disease. The epilepsy phenotype was very mild and was characterized by focal seizures that often remitted spontaneously or were easily controlled by AEDs. The family was first considered to have benign familial epilepsy, delaying diagnosis. Clinical variability within the family caused a number of family members to escape from the strict man-made diagnostic criteria for TSC. Physicians should be aware that patients who do not fulfill the diagnostic criteria may still have TSC.

In this family, no cortical tubers were detected by high quality MRI imaging. However, 60% of mutation carriers had seizures. Further investigations are required to determine the origin of the seizures in these individuals.

We have identified 10 new families with changes at codon 905 of *TSC2*. The R905Q change constitutes an example of a TSC gene mutation associated with a low probability of developing a severe TSC phenotype, which can be helpful in counseling. However, the possibility of clinical variation should still be considered.

The finding of a more severe phenotype associated with the sporadic R905W mutation as compared to the familial R905Q mutation was consistent with the functional studies, since R905W was less stable or active in 2 assays. Our findings support the observation that familial TSC, even when it is due to a *TSC2* mutation, is less severe than sporadic TSC [24, 31].

This report illustrates how difficult establishing a definite diagnosis of TSC can be. Functional studies can help to understand the impact of a given mutation on hamartin/tuberin function. Therefore, refining the diagnosis of TSC based on mutation analysis and functional studies can contribute to counseling individual patients and families.

Acknowledgements

The authors would like to thank the patients and their families for their excellent collaboration. They also wish to thank Ms Marie-Hélène Seni for technical assistance and Dr G. Li for help with the ophthalmological assessment of patients in Family A. This study was supported in part by a grant from the Canadian Institutes of Health Research (CIHR) to EA and by a grant from the National Institutes of Health (NIH), National Institute of Neurological Disorders and Stroke (grant #31535) to DJK. MP received funding from the 'Fonds National de la Recherche Scientifique' (Belgium) and the Interuniversity Attraction Poles Program P5/19 of the Belgian Federal Science Policy Office. AJ was funded by the Fondation Belge de la Vocation/Belgische Stichting Roeping and the Savoy Foundation for Epilepsy Research. MDD received funding from the National Epifellows Foundation. GG received a scholarship (Junior 1) from the 'Fonds de la Recherche en Santé du Quebéc' (FRSQ).

References

1. Gomez, M.R., J.R. Sampson, and V.H. Whittemore, eds. *Tuberous sclerosis complex*. 3rd ed. Developmental perspectives in psychiatry, ed. J.C. Harris. 1999, Oxford University Press: New York. 340.
2. Hunt, A. and J. Dennis, *Psychiatric disorder among children with tuberous sclerosis.* Dev Med Child Neurol, 1987. **29**(2): p. 190-8.
3. Smalley, S.L., et al., *Autism and tuberous sclerosis.* J Autism Dev Disord, 1992. **22**(3): p. 339-55.
4. Joinson, C., et al., *Learning disability and epilepsy in an epidemiological sample of individuals with tuberous sclerosis complex.* Psychol Med, 2003. **33**(2): p. 335-44.

5. Lewis, J.C., et al., *Genotype and psychological phenotype in tuberous sclerosis.* J Med Genet, 2004. **41**(3): p. 203-7.
6. Shepherd, C.W., et al., *Causes of death in patients with tuberous sclerosis.* Mayo Clin Proc, 1991. **66**(8): p. 792-6.
7. Sampson, J.R., et al., *Genetic aspects of tuberous sclerosis in the west of Scotland.* J Med Genet, 1989. **26**(1): p. 28-31.
8. Osborne, J.P., A. Fryer, and D. Webb, *Epidemiology of tuberous sclerosis.* Ann N Y Acad Sci, 1991. **615**: p. 125-7.
9. Gunther, M. and L.S. Penrose, *The genetics of epiloia.* J Genet, 1935. **31**: p. 413-430.
10. Young, J.M., et al., *A mutation screen of the TSC1 gene reveals 26 protein truncating mutations and 1 splice site mutation in a panel of 79 tuberous sclerosis patients.* Ann Hum Genet, 1998. **62 (Pt 3)**: p. 203-13.
11. Kandt, R.S., et al., *Linkage of an important gene locus for tuberous sclerosis to a chromosome 16 marker for polycystic kidney disease.* Nat Genet, 1992. **2**(1): p. 37-41.
12. Fryer, A.E., et al., *Evidence that the gene for tuberous sclerosis is on chromosome 9.* Lancet, 1987. **1**(8534): p. 659-61.
13. Haines, J.L., et al., *Localization of one gene for tuberous sclerosis within 9q32-9q34, and further evidence for heterogeneity.* Am J Hum Genet, 1991. **49**(4): p. 764-72.
14. Short, M.P., et al., *Clinical findings and linkage studies in familial tuberous sclerosis.* Ann N Y Acad Sci, 1991. **615**: p. 380-1.
15. van Slegtenhorst, M., et al., *Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34.* Science, 1997. **277**(5327): p. 805-8.
16. Consortium, E.C.T.S., *Identification and characterization of the tuberous sclerosis gene on chromosome 16.* Cell, 1993. **75**: p. 1305-15.
17. Cheadle, J.P., et al., *Molecular genetic advances in tuberous sclerosis.* Hum Genet, 2000. **107**(2): p. 97-114.
18. Dabora, S.L., et al., *Mutational analysis in a cohort of 224 tuberous sclerosis patients indicates increased severity of TSC2, compared with TSC1, disease in multiple organs.* Am J Hum Genet, 2001. **68**(1): p. 64-80.
19. Green, A.J., M. Smith, and J.R. Yates, *Loss of heterozygosity on chromosome 16p13.3 in hamartomas from tuberous sclerosis patients.* Nat Genet, 1994. **6**(2): p. 193-6.
20. Henske, E.P., et al., *Loss of heterozygosity in the tuberous sclerosis (TSC2) region of chromosome band 16p13 occurs in sporadic as well as TSC-associated renal angiomyolipomas.* Genes Chromosomes Cancer, 1995. **13**(4): p. 295-8.
21. Henske, E.P., et al., *Allelic loss is frequent in tuberous sclerosis kidney lesions but rare in brain lesions.* Am J Hum Genet, 1996. **59**(2): p. 400-6.
22. van Slegtenhorst, M., et al., *Interaction between hamartin and tuberin, the TSC1 and TSC2 gene products.* Hum Mol Genet, 1998. **7**(6): p. 1053-7.
23. Tee, A.R., et al., *Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling.* Proc Natl Acad Sci U S A, 2002. **99**(21): p. 13571-6.
24. Au, K.S., et al., *Genotype/phenotype correlations in familial tuberous sclerosis cases (abstract 2339).* Am J Hum Genet, 2003.

25. van Slegtenhorst, M., et al., *Mutational spectrum of the TSC1 gene in a cohort of 225 tuberous sclerosis complex patients: no evidence for genotype-phenotype correlation.* J Med Genet, 1999. **36**(4): p. 285-289.
26. Roach, E.S., M.R. Gomez, and H. Northrup, *Tuberous sclerosis complex consensus conference: revised clinical diagnostic criteria.* J Child Neurol, 1998. **13**(12): p. 624-8.
27. Gustafson, S., et al., *Parameters affecting the yield of DNA from human blood.* Anal Biochem, 1987. **165**(2): p. 294-9.
28. Lathrop, G.M., et al., *Multilocus linkage analysis in humans: detection of linkage and estimation of recombination.* Am J Hum Genet, 1985. **37**(3): p. 482-98.
29. Choy, Y.S., et al., *Superiority of denaturing high performance liquid chromatography over single-stranded conformation and conformation-sensitive gel electrophoresis for mutation detection in TSC2.* Ann Hum Genet, 1999. **63 (Pt 5)**: p. 383-91.
30. Roberts, P.S., et al., *Denaturing high-performance liquid chromatography (DHPLC) is a highly sensitive, semi-automated method for identifying mutations in the TSC1 gene.* J Biochem Biophys Methods, 2001. **47**(1-2): p. 33-7.
31. Sancak, O., et al., *Mutational analysis of the TSC1 and TSC2 genes in a diagnostic setting: genotype-phenotype correlations and comparison of diagnostic DNA techniques in Tuberous Sclerosis Complex.* submitted, 2005.
32. Nellist, M., et al., *Distinct effects of single amino-acid changes to tuberin on the function of the tuberin-hamartin complex.* Eur J Hum Genet, 2005. **13**(1): p. 59-68.
33. Nellist, M., et al., *TSC2 missense mutations inhibit tuberin phosphorylation and prevent formation of the tuberin-hamartin complex*, in *Hum Mol Genet*. 2001. p. 2889-98.
34. Jones, A.C., et al., *Comprehensive mutation analysis of TSC1 and TSC2, and phenotypic correlations in 150 families with tuberous sclerosis.* Am J Hum Genet, 1999. **64**(5): p. 1305-15.
35. Gleeson, J.G., et al., *Characterization of mutations in the gene doublecortin in patients with double cortex syndrome.* Ann Neurol, 1999. **45**(2): p. 146-53.
36. Zhang, H., et al., *Mutational analysis of TSC1 and TSC2 genes in Japanese patients with tuberous sclerosis complex.* J Hum Genet, 1999. **44**(6): p. 391-6.
37. Jones, A.C., et al., *Molecular genetic and phenotypic analysis reveals differences between TSC1 and TSC2 associated familial and sporadic tuberous sclerosis.* Hum Mol Genet, 1997. **6**(12): p. 2155-61.
38. Yamamoto, T., et al., *Novel TSC1 and TSC2 mutations in Japanese patients with tuberous sclerosis complex.* Brain and Development, 2002. **24**(4): p. 227-230.
39. Yamashita, Y., et al., *Analysis of all exons of TSC1 and TSC2 genes for germline mutations in Japanese patients with tuberous sclerosis: report of 10 mutations.* Am J Med Genet, 2000. **90**(2): p. 123-6.
40. Niida, Y., et al., *Analysis of both TSC1 and TSC2 for germline mutations in 126 unrelated patients with tuberous sclerosis.* Hum Mutat, 1999. **14**(5): p. 412-22.
41. Kwiatkowska, J., et al., *Comprehensive mutational analysis of the TSC1 gene: observations on frequency of mutation, associated features, and nonpenetrance.* Ann Hum Genet, 1998. **62 (Pt 4)**: p. 277-85.

42. Povey, S., et al., *Two loci for tuberous sclerosis: one on 9q34 and one on 16p13.* Ann Hum Genet, 1994. **58 (Pt 2)**: p. 107-27.
43. Au, K.S., et al., *Germ-line mutational analysis of the TSC2 gene in 90 tuberous-sclerosis patients.* Am J Hum Genet, 1998. **62**(2): p. 286-94.
44. Beauchamp, R.L., et al., *Exon scanning of the entire TSC2 gene for germline mutations in 40 unrelated patients with tuberous sclerosis.* Hum Mutat, 1998. **12**(6): p. 408-16.
45. Gilbert, J.R., et al., *Mutation and polymorphism analysis in the tuberous sclerosis 2 (TSC2) gene.* Neurogenetics, 1998. **1**(4): p. 267-72.
46. Wilson, P., et al., *Novel mutations detected in the TSC2 gene from both sporadic and familial TSC patients.* Hum. Mol. Genet., 1996. **5**(2): p. 249-256.
47. Humphrey, A., et al., *Monozygotic twins with tuberous sclerosis discordant for the severity of developmental deficits.* Neurology, 2004. **62**(5): p. 795-8.
48. Martin, N., et al., *Discordant clinical manifestations in monozygotic twins with the identical mutation in the TSC2 gene.* Clin Genet, 2003. **63**(5): p. 427-30.
49. Verhoef, S., et al., *Somatic mosaicism and clinical variation in tuberous sclerosis complex.* Lancet, 1995. **345**(8943): p. 202.
50. Ewalt, D.H., et al., *Renal lesion growth in children with tuberous sclerosis complex.* J Urol, 1998. **160**(1): p. 141-5.
51. Khare, L., et al., *A novel missense mutation in the GTPase activating protein homology region of TSC2 in two large families with tuberous sclerosis complex.* J Med Genet, 2001. **38**(5): p. 347-349.
52. O'Connor, S.E., et al., *A family with seizures and minor features of tuberous sclerosis and a novel TSC2 mutation.* Neurology, 2003. **61**(3): p. 409-412.
53. Mayer, K., et al., *Characterisation of a novel TSC2 missense mutation in the GAP related domain associated with minimal clinical manifestations of tuberous sclerosis.* J Med Genet, 2004. **41**: p. e64.

Appendix: Case Reports Family A

A younger brother of the maternal grandfather, III-11, a 74-year-old man, has some well defined white skin spots, but never had seizures.

His daughter, IV-20, a 43-year-old woman, had 5 to 10 focal seizures during one week at age five years, fully controlled after treatment was started. Medication was discontinued at age 11 and she remained seizure free. She has multiple small hypomelanotic macules and confetti spots (CS). MRI of the brain showed TSC related white matter lesions in the left frontal lobe. Cardiac and abdominal ultrasound as well as ophthalmological examination, were entirely normal.

Her 22-year-old daughter, V-22, had several hypomelanotic macules and confetti spots. She never had seizures and was a good student. A full diagnostic work-up for TSC remained negative.

Her 17-year-old son, V-23, was born after normal pregnancy and had two episodes of apnea shortly after birth. Convulsions occurred on days 2 and 3. He was treated with phenobarbital and remained seizure free after treatment was discontinued. At age 13 years, he had a generalized tonic-clonic seizure for which he was treated with valproic acid. He had another generalized attack at age 15, after the medication had been reduced. A single focal seizure occurred at age 16. He also had migraine. At school, he started having difficulties after he began to have seizures and had to repeat grade 9. He had multiple hypomelanotic macules and several confetti spots. MRI of the brain showed left parietal white matter lesions compatible with perinatal damage, as well as TSC-related white matter lesions in both frontal lobes. He had a single small renal cyst. Eye exam and cardiac ultrasound were normal.

Another brother of the proband's maternal grandfather, III-13, a 71-year-old man, did not have seizures or skin lesions based on history.

His daughter, IV-26, a 44-year-old woman, started having generalized seizures at age 15 months and is partially controlled with antiepileptic drugs. She has mild cognitive impairment and attended a special school.

Her daughter, V-25, a 24-year-old woman, has had focal seizures with secondary generalization since age 17 years, partially controlled with AEDs. In addition, she has non epileptic seizures as well as migraine. She had several hypomelanotic macules and confetti spots. Full scale IQ ratings were in the borderline range. Brain imaging showed a single left frontal partly calcified SEGA with focal ventricular enlargement due to compression of the left Foramen of Monroe, as well as one TSC-related white matter lesion in the left frontal lobe. Cardiac and abdominal ultrasound as well as eye exam were normal.

Her 9-year-old daughter, VI-4, has two hypomelanotic macules and several confetti spots, but further work-up was negative.

The youngest sister of the proband's maternal grandfather, III-21, a 60-year-old woman, has several hypomelanotic macules and confetti spots, as well as a TSC related white matter lesion in the right frontal lobe. She never had seizures and further work-up was normal.

Her daughter, IV-34, a 42-year-old woman, developed seizures at age four years. Attacks were focal with secondary generalization, and could be precipitated by stress or strong emotions. Seizures disappeared at age seven and treatment was discontinued at age 11. She studied until grade 11 and had some difficulties with language. She has several HMM and CS. Brain, kidneys, heart and eyes were normal.

Her 22-year-old daughter, V-27, started having focal attacks with secondary generalization at age 3.5 years. Seizures were difficult to control but finally responded to clonazepam and valproic acid. She then showed developmental regression and self-mutilating behavior, which improved when medication was switched to carbamazepine. She has been well controlled since and did well in her studies. She has several HMM and CS, two subependymal nodules (SEN) projecting in the left and right lateral ventricle, and a single renal AML.

A first cousin of the proband's maternal grandfather, III-5, a 54-year-old woman, had focal seizures with secondary generalization between ages 20 and 40, and afterwards remained seizure free without treatment. She has two hypomelanotic macules.

Her daughter, IV-3, a 32-year-old woman, has several HMM and CS but never had seizures.

Her 10-year-old daughter, V-1, had refractory epilepsy. She started having focal seizures at age 22 months and would have up to 15 attacks per day. Partial control was obtained with phenytoin and clobazam. Early development was normal but slowed after the onset of seizures. She has several hypomelanotic macules and confetti spots. Brain CT and MRI, cardiac and abdominal ultrasound, as well as eye examination were normal.

FUNCTIONAL ASSAYS AS A DIAGNOSTIC TOOL IN TUBEROUS SCLEROSIS COMPLEX

Ozgur Sancak[1], Mark Nellist[1], Miriam Goedbloed[1], Marja Wessels[1], Anneke Maat-Kievit[1], Marieke Baars[2], Charlotte Dommering[2], Ans van den Ouweland[1], Dicky Halley[1]*

[1] Department of Clinical Genetics, Erasmus Medical Centre, Rotterdam, the Netherlands
[2] Department of Clinical and Human Genetics, VU Medical Centre, Amsterdam, the Netherlands

* To whom correspondence should be addressed: Dr. Dicky Halley, Department of Clinical Genetics, Erasmus Medical Centre, Dr. Molewaterplein 50, 3015 GE Rotterdam, the Netherlands, Tel: +31 10 4087197; Fax: +31 10 4089489; E-mail: d.halley@erasmusmc.nl

Article submitted for publication

Running title: Functional assays in Tuberous Sclerosis Complex
Keywords: Tuberous Sclerosis Complex, functional assays, non-truncating mutations, mild phenotype in TSC, mutation analysis

ABSTRACT

Tuberous Sclerosis Complex (TSC) is an autosomal dominant disorder caused by mutations in either the *TSC1* or *TSC2* gene. A pathogenic mutation can be identified in approximately 85% of definite TSC cases. In the majority of cases, the result of the mutation analysis is straightforward. However, establishing the pathogenicity of a non-truncating sequence change to confirm or ascertain the diagnosis, particularly where there is insufficient genetic or clinical data, can be difficult or impossible. Here we describe two families with index cases not fulfilling the diagnostic clinical criteria of definite TSC. Mutation analysis resulted in the identification of two non-truncating changes, one from each parent, in both index patients. We characterized these changes using functional assays and identified pathogenic mutations in both families facilitating the diagnosis of affected family members. Here we discuss the use of functional assays as a diagnostic tool and the consequences of the identification of pathogenic mutations in these families.

INTRODUCTION

Tuberous Sclerosis Complex (TSC, MIM 191100) is an autosomal dominant disorder characterized by hamartomas and hamartias in multiple organs and tissues, affecting 1 in 6000-10000 individuals [1]. TSC is caused by mutations in either the *TSC1* gene on chromosome 9q34 or the *TSC2* gene on chromosome 16p13 [2, 3]. In approximately two-thirds of the cases TSC is caused by a *de novo* mutation indicating a high spontaneous mutation rate.

A broad spectrum of mutations has been identified in both genes [2-8]. There are recurrent mutations, however, many of the mutations are unique to the families they are identified in. The majority of TSC patients have a mutation in the *TSC2* gene. In *de novo* cases, mutations in the *TSC2* gene are found two to ten times more often than *TSC1* mutations [4, 7-10]. In contrast, in multi-generation, informative families segregating TSC, approximately half show linkage to *TSC1* (9q34) and half to *TSC2* (16p13) [11].

Loss of heterozygosity across the *TSC1* and *TSC2* loci in TSC-associated lesions indicates that *TSC1* and *TSC2* are tumour suppressor genes [12]. The *TSC1* and *TSC2* gene products, hamartin and tuberin, form a complex [13] that activates the GTPase activity of rheb, and inhibits the rheb-GTP dependent stimulation of cell growth through mTOR [14]. It is now possible to study functional aspects of the tuberin-hamartin complex *in vitro*, including complex formation, *in vitro* rheb GAP activity, and the phosphorylation status of p70S6K and S6, the downstream effectors of the signalling pathway through mTOR [15].

TSC diagnosis is based on clinical criteria that are categorized as major and minor features [16]. The presence of two major features or one major and two minor features is sufficient for a definite diagnosis. However, the phenotypic expression of TSC is highly variable, even within families, making it difficult to establish a diagnosis in some cases. In recent years, mutation analysis has become an additional diagnostic tool in familial TSC as well as in sporadic cases.

The utility of mutation analysis for diagnosis and genetic counselling requires the correct differentiation between (rare) polymorphisms and pathogenic mutations, which may be very complicated for non-truncating sequence changes in particular. In familial cases where a non-truncating nucleotide change cosegregates with TSC or in cases where key relatives are not available for testing, this distinction cannot be made on genetic grounds only.

Here, we describe two families with index cases not fulfilling the diagnostic clinical criteria of definite TSC. The index case of family 1 had cardiac rhabdomyomas and deceased postpartum, without any other signs or symptoms of TSC. The index case of family 2 had epilepsy, hypomelanotic macules and dental pits also not fulfilling the criteria for a definite diagnosis of TSC. In both index cases two non-truncating changes in the *TSC2* gene, one from each parent, were identified. We characterized the changes using genetic and biochemical analyses and determined the disease-causing mutations in both families. The consequences of the identification of pathogenic mutations in these families and the diagnostic use of functional analyses in the characterization of previously unpublished nucleotide changes will be discussed.

MATERIALS AND METHODS

DNA was extracted from peripheral blood using standard techniques. Mutation analysis was performed using a combination of single-strand conformational polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE) and direct sequencing. Both the *TSC1* and the *TSC2* genes were analysed completely. To detect larger rearrangements in the *TSC2* gene, in addition to Southern blotting and fluorescence in situ hybridisation (FISH) [8], a Multiplex Ligation-dependent Probe Amplification (MLPA) assay (MRC, Holland) was performed . Numbering of the sequence changes is according to the accession numbers: AF013168 (*TSC1*) and X75621 (*TSC2*).

Amino acid substitutions were evaluated using BLOSUM 62, PAM 250 and Grantham score matrices. In order to investigate if any of the nucleotide changes had an effect on RNA splicing, these were tested using three different splice-site prediction programs (www.cbs.dtu.dk/services/NetGene2, www.genet.sickkids.on.ca/~ali/splicesitefinder.html, and www.fruitfly.org/ seq_tools/splice.html).

Full-length *TSC1* and *TSC2* expression constructs have been described previously [15, 17]. Expression constructs encoding tuberin variants were derived by site-directed mutagenesis using the Stratagene QuickChange kit. All constructs were sequenced completely.

Polyclonal rabbit antisera specific for human tuberin and hamartin have been described previously [18]. All other antibodies were purchased from Cell Signaling Technology.

Transfections, immunocytochemistry, immunoblotting, immunoprecipitation assays and the *in vitro* assay of rheb GTPase activity were performed as described previously [15].

RESULTS

Cases and Family Characteristics

Family 1:

The index case (figure 1a, III:3) of family 1 was diagnosed with a solitary rhabdomyoma in the right ventricle of the heart and signs of heart failure *in utero* and deceased postpartum. Post-mortem examination showed no other findings indicative of TSC. Both parents underwent a full clinical evaluation. Dermatological, cardiological, ophthalmological, neurological as well as radiological examinations were negative for signs and symptoms of TSC with the exception of one nail groove and one hypomelanotic macule in the father (II:3), which was insufficient for a diagnosis of TSC. The older brother of the index case (III:2) was healthy, but was not investigated further. At the first trimester of the third pregnancy, mutation analysis of the index case had not been completed and prenatal DNA testing could not be offered. Subsequent fetal echocardiography during pregnancy revealed no heart tumours. The pregnancy resulted in the birth of a healthy boy (III:4) but follow up of the family was not possible.

Family 2:

The index case (figure 1b, III:4) of family 2 had five hypomelanotic macules and multiple dental pits accompanied by epilepsy and EEG abnormalities. The patient was diagnosed with 'probable TSC'. The mother (II:10) of the index case had multiple dental pits, which is considered a 'minor feature' of TSC, and two small calcifications in the nucleus caudatus on CT scan, which are not typical for TSC. MR imaging was performed and showed no additional abnormalities. Her brother (II:11), deceased at the age of 13, had mental retardation, epilepsy, neurologic and ophthalmologic problems. He died of status epilepticus. The father (II:9) had one 2-cm cyst in the left kidney and an ash leaf-shaped area of hypopigmentation. Some time after the referral of the index case to our centre for mutation analysis, the older half sister (III:1) of the index case by the same father was seen by another genetic counsellor. She presented at the age of 12 years with epileptic seizures. A full diagnostic work-up showed that she (III:1) had cortical tubers and seven hypomelanotic macules, fulfilling the diagnostic criteria for definite TSC. She had no further signs or symptoms related to TSC. Her mother (II:8) was found to have multiple dental pits and ten irregular areas of hypopigmentation, atypical for TSC, without further signs of the disease.

See figure 1 next page

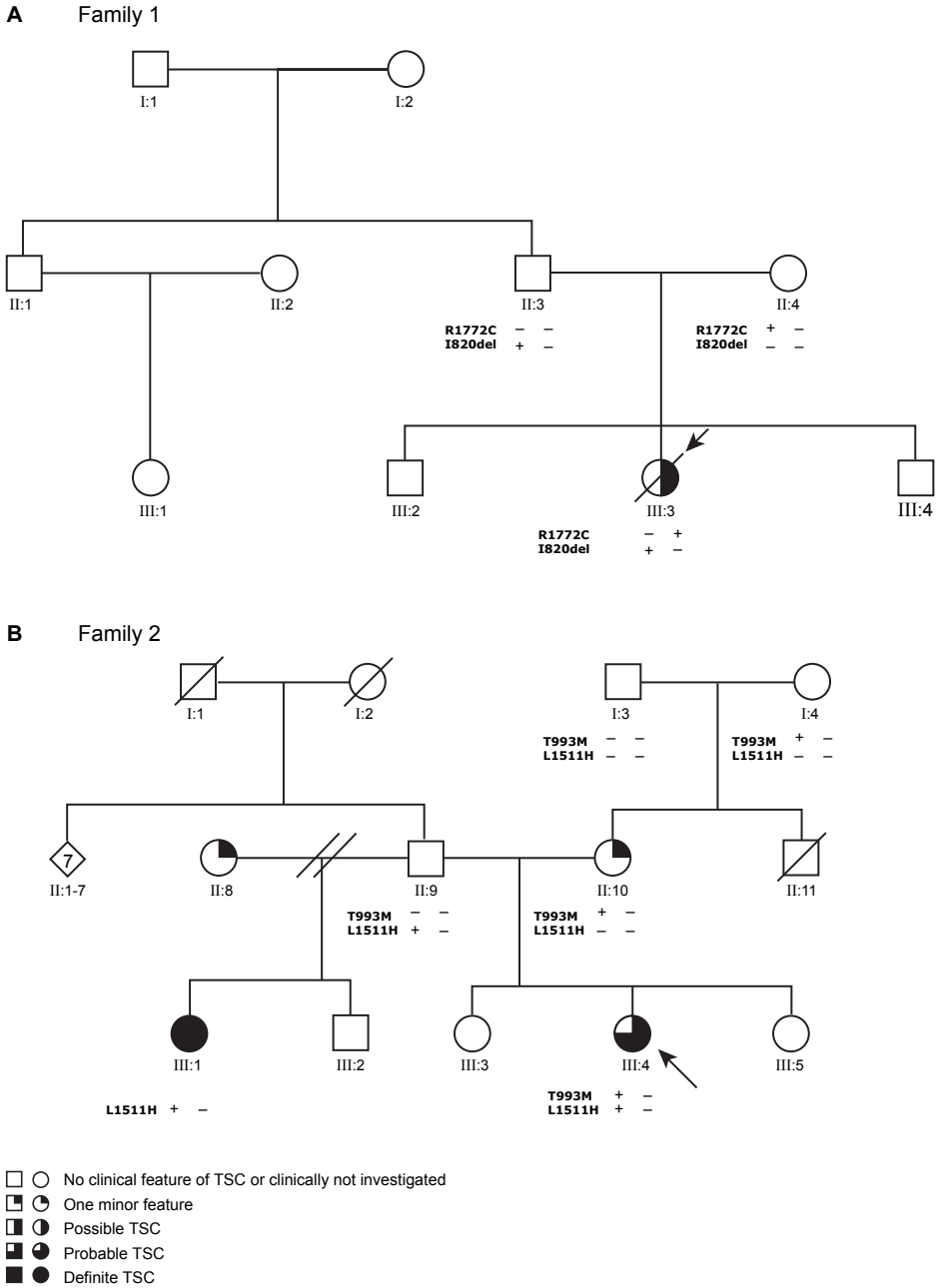


Figure 1: Pedigrees of the investigated families, family 1(a) and family 2(b). Arrows indicate the index cases. Clear symbols indicate no signs or symptoms of TSC; ¼ full symbols indicate one minor feature of TSC, ½ full symbols indicate possible, ¾ full symbols indicate probable and full symbols indicate definite TSC. Results of the mutation analysis are indicated where available.

Mutation analysis

The index case of family 1 was found to be heterozygous for both 2476delATC (deletion of amino acid isoleucine at position 820, I820del) (of paternal origin) and 5332C>T (arginine to cysteine amino acid substitution at position 1772, R1772C) (of maternal origin) in the *TSC2* gene. No DNA was available from other relatives. In the index case of family 2, the 2996C>T (threonine to methionine amino acid substitution at position 993, T993M) and the 4550T>A (leucine to histidine amino acid substitution at position 1511, L1511H) heterozygous missense changes in the *TSC2* gene were identified. T993M was of maternal origin and L1511H was of paternal origin. The maternal grandparents (I:3 and I:4) of the index case were also tested for the aforementioned nucleotide changes and the maternal grandmother (I:4) was found to be heterozygous for the missense change T993M. Subsequently, a complete mutation screen of both genes was performed on DNA of the half sister of the index case and she was found to be heterozygous for L1511H only. We compared the allele ratios of the index cases and (grand-) parents by direct sequencing of leukocyte DNA and found no evidence for somatic mosaicism of the (grand-) parents in either family. None of the changes showed an effect on splicing according to the splice-site prediction programs used.

Amino acid conservation

The amino acids I820, T993, L1511 and R1772 are all conserved in tuberin orthologues in different species including mus musculus, rattus norvegicus, fugu rubripes, gallus gallus and xenopus tropicalis. The amino acid substitutions were evaluated using the BLOSUM 62, PAM250 and Grantham score matrices. The BLOSUM 62 and PAM250 matrices gave negative results and the Grantham score matrix gave high scores for the three amino acid substitutions indicating that these changes were non-conservative changes. Therefore, the *in silico* analyses suggested that any of these non-truncating nucleotide changes might have functional implications and might be a pathogenic mutation (table 1).

Family	Codon	Amino Acid Substitution	BLOSUM 62	PAM 250	Grantham score
Family 1	820	del I	N/A	N/A	N/A
Family 1	1772	R>C	-3	-4	180
Family 2	993	T>M	-1	-1	81
Family 2	1511	L>H	-3	-2	99

Table 1: Scores of the BLOSUM 62, PAM 250 and Grantham score matrices. Theoretical scores for BLOSUM 62 are between -4 and 11, and for PAM 250 between -8 and 17. The more negative the scores for BLOSUM 62 and PAM 250 matrices, the less conserved the amino acid change. Theoretical Grantham scores are between 5 and 205 and the higher the scores, the less conserved the amino acid change. (N/A: not applicable)

Functional Analysis

Tuberin variants containing I820del, R1772C, T993M and L1511H changes were compared with wild-type tuberin and a known pathogenic missense variant, R611Q, using assays of tuberin-hamartin complex formation, rheb GAP activity of the complex and the phosphorylation status of p70S6K (S6K) and S6 (figure 2).

I820del clearly reduced coimmunoprecipitation of the tuberin-hamartin complex (figure 2a), while R1772C, T993M and L1511H did not have visible effects indicating that the I820del tuberin variant can less efficiently bind to and form a (stable) complex with hamartin.

To investigate whether the tuberin amino acid changes affected the GTPase rate of rheb, we assayed the rhebGAP activity of the tuberin-hamartin complexes. As shown in figure 2b, immunoprecipitated wild-type complex facilitated the GTPase activity of rheb. The GAP activities of tuberin I820del, L1511H and R611Q variants were reduced compared to those of the wild-type tuberin, and the R1772C and T993M variants.

We overexpressed wild-type tuberin or the tuberin variants together with hamartin and S6K in human embryonal kidney 293 cells, and compared the phosphorylation status of the linker domain (T389) of exogenous S6K by immunoblotting. As shown in figure 2c, coexpression of hamartin and either wild-type tuberin or the R1772C and T993M variants inhibited S6K phosphorylation. The inhibition of S6K phosphorylation by the I820del, the L1511H and the R611Q variants, on the other hand, was reduced.

Tsc2 - / - mouse embryo fibroblasts (MEFs), which have constitutively phosphorylated S6, were transfected with tuberin or tuberin variants together with hamartin to determine their ability to reduce S6 phosphorylation. We assayed the phosphorylation of S6 in transfected, serum-starved cells by double-label immunofluorescent microscopy as described previously (figure 2d) [15]. Wild-type tuberin as well as the R1772C and the T993M tuberin variants were able to suppress S6 phosphorylation in more than 90% of the cells when coexpressed with hamartin. In contrast, the I820del, the L1511H and the R611Q variants could not inhibit S6 phosphorylation efficiently compared to wild-type tuberin or the R1772C and T993M variants.

We classified the I820del and L1511H changes as pathogenic mutations and R1772C and T993M as non-pathogenic amino acid substitutions.

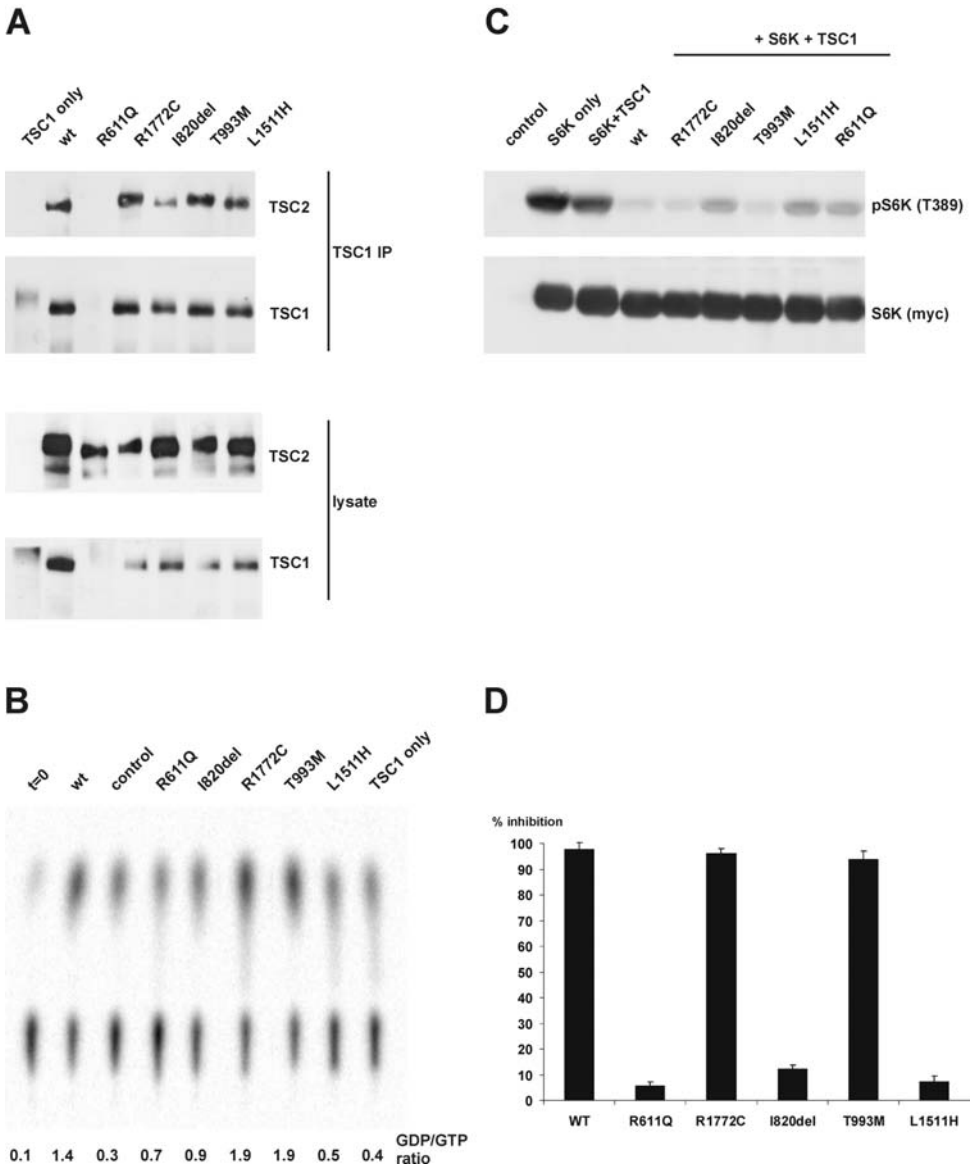


Figure 2: Results of the functional assays. (a) Interaction between hamartin (TSC1) and tuberin (TSC2) variants. TSC1-TSC2 complexes were immunoprecipitated with TSC1-specific antibodies from COS cells expressing exogenous TSC1 and wild-type TSC2 (wt) or TSC2 variants. (b) In vitro rhebGAP activity of immunoprecipitated TSC1-TSC2 complexes. Rheb-bound GDP/GTP ratios were determined after 90 minutes incubation with the wild-type TSC1-TSC2 complex (wt), protein A beads only (control), TSC1 only, or TSC1-TSC2 variant complexes. (c)TSC2-dependent inhibition of S6K-T389 phosphorylation. S6K, TSC1 and wild-type TSC2 (wt), or TSC2 variants, were co-expressed in HEK293 cells. Phosphorylation of S6K at T389 position was determined by Western blotting. (d) Inhibition of S6 phosphorylation. Average inhibition of S6 phosphorylation in *Tsc2* - / - mouse embryo fibroblasts transfected with TSC1 and wild-type TSC2 or TSC2 variants from three different experiments are shown.

DISCUSSION

We demonstrated that our mutation analysis strategy enables us to determine pathogenic mutations in approximately 85% of definite TSC patients [8]. However, in cases where there is insufficient clinical and/or genetic data, the establishment of the pathogenicity of identified genetic variants, particularly of non-truncating changes, is difficult. In selected cases, where amino acid matrix scores for the identified amino acid substitutions suggest biochemical consequences and where DNA is available from family members, we apply functional assays to determine the nature of these sequence variants.

Here, we describe two families with symptomatic index patients, who did not fulfil the criteria for definite TSC, and apparently unaffected parents, four of which had insufficient or ambiguous signs of the disease after clinical evaluation (figure 1). Both index patients inherited two non-truncating nucleotide changes, one from each parent.

We characterized the effects of the identified changes on the function of the tuberin-hamartin complex in order to determine whether any of these changes were disease-causing mutations. The I820, R1772, T993 and L1511 amino acids are all conserved in tuberin orthologues and amino acid substitution matrices indicated that the R1772C, T993M and L1511H amino acid changes might all have functional implications. Functional analysis of these changes showed that the I820del variant formed a complex with hamartin less efficiently than the R1772C, T993M, L1511H variants or the wild-type tuberin, suggesting that the I820del variant might be pathogenic. Both the I820del and L1511H variants had less *in vitro* GAP activity towards rheb and, consistent with a dysregulating effect on the mTOR signalling pathway, both variants were less efficient in inhibiting S6K and S6 phosphorylation. These results showed that the non-truncating changes I820del and L1511H had similar effects to the known pathogenic missense mutation R611Q and disrupted the function of the tuberin-hamartin complex *in vitro.* Therefore, we classified I820del and L1511H as pathogenic mutations and R1772C and T994M as rare polymorphisms.

Confirmation of the pathogenicity of the I820del mutation meant that a diagnosis of TSC could be assigned to the index patient of family 1, who had only cardiac rhabdomyoma *in utero*. Rhabdomyoma is the most common foetal and neonatal cardiac tumour. It can be associated with several genetic disorders, but TSC has been implicated in as many as two-thirds of the cases [19]. Although the majority of these cases appear to be sporadic, careful evaluation of the parents for signs and symptoms of TSC is recommended. In family 1, the father's very mild presentation of the disease was not even sufficient for a diagnosis of 'possible TSC'. Although somatic mosaicism was not rigorously excluded, since only leukocyte DNA was analysed, the recurrence risk for this couple is considered to be 50%. Since the other children (figure 1a; III:2 and III:4) were not available for DNA testing or clinical evaluation, at the moment it cannot be excluded that either one of them is also carrying the familial mutation.

After mutation analysis of the index patient's DNA in family 2 resulting in the identification of both

T993M and L1511H, her half sister (III:1 in fig 1b) was diagnosed with definite TSC on the basis of clinical criteria. The half sister's DNA was subjected to a complete mutation screen of both genes and she was shown to carry L1511H only. This excluded T993M as the common cause of disease in the half-sisters, but it did not establish the pathogenicity of L1511H, which could be a cosegregating rare polymorphism. The presence of multiple dental pits in both mothers of the affected individuals in family 2 (II:8 and II:10) might indicate that TSC in individuals III:1 and III:4 are caused by two different mutations inherited from their respective mothers. However, the functional tests confirmed the pathogenic nature of L1511H.

BLOSUM 62, PAM 250 and Grantham score matrices give indications if an amino acid substitution might have a significant biochemical effect. Although these matrices might give a clue, the same amino acid substitution might have different consequences depending on the surrounding amino acids and the domain it is located at. For example, while R>Q amino acid substitution results in complete loss of function at position 611 of tuberin, it has no detectable functional consequences at position 367 [15] (and unpublished data, 2005). The use of these functional assays to differentiate between polymorphisms and pathogenic mutations facilitates not only the identification of pathogenic mutations in difficult cases but also might help identify functionally important domains and explain in part the phenotypic heterogeneity. Comparison of the effects of I820del and L1511H suggests that these two amino acids are involved in different aspects of tuberin function. I820del disrupts the formation of a functional hamartin-tuberin complex, makes tuberin less stable, or causes a conformational change prohibiting hamartin-tuberin binding. L1511H, on the other hand, does not disrupt complex formation. However, the complex formed with this tuberin variant has reduced GAP activity and is less able to inhibit phosphorylation of the downstream effectors of the mTOR signalling pathway. It should be noted that L1511 may be located within the GAP domain, the borders of which have not been defined consistently [3, 4, 20] (http://www.expasy. ch/cgi-bin/nicesite.pl?PS50085, http://www.sanger.ac.uk/cgi-bin/Pfam/getacc ?PF02145).

The use of functional assays in characterization of non-truncating nucleotide changes is limited by time and resources. To minimize repetition and unnecessary work, international collaboration through an up-to-date database with all the mutations and polymorphisms identified in the *TSC1* and the *TSC2* genes worldwide is necessary for diagnostic facilities as well as for researchers.

Genetic testing was not included in the revised diagnostic criteria of TSC. However, an individual with a pathogenic mutation in the *TSC1* or *TSC2* gene who does not fulfil the clinical diagnostic criteria, which is the case for both of the fathers in these two families, has up to a 50% risk of having affected offspring and an increased risk of developing various TSC associated lesions. Such an individual should carefully be followed-up since while some TSC associated lesions do not appear until a certain age, others may disappear with age, complicating diagnosis.

Our results illustrate the phenotypic variability of TSC, even within families. A non-truncating change identified in an index patient as well as in a "seemingly unaffected" parent is not necessarily an innocent polymorphism. Functional assays have proven to be an important diagnostic tool

that complements mutation analysis. Identification of pathogenic mutations in the families we described here enables not only genetic counselling and prenatal testing for future pregnancies but also diagnosis of affected family members for critical clinical care.

ACKNOWLEDGMENTS

Financial support was provided by the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO), the Nationaal Epilepsie Fonds and the Department of Clinical Genetics. Dr H Onda (Brigham and Women's Hospital, USA) is thanked for supplying the *Tsc2* -/- MEFs. Dr M van Slegtenhorst (Fox Chase Cancer Center, USA) and Dr T Nobukini (Friedrich Miescher Institute, Switzerland) are thanked for supplying rheb and S6K expression constructs respectively. We thank the family members who contributed to this study.

REFERENCES

1. Gomez M, Sampson J, Whittemore V, eds. *The tuberous sclerosis complex.* Oxford, England: Oxford University Press, 1999.
2. van Slegtenhorst M, de Hoogt R, Hermans C*, et al.* Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. *Science* 1997;**277**(5327):805-8.
3. Identification and characterization of the tuberous sclerosis gene on chromosome 16. The European Chromosome 16 Tuberous Sclerosis Consortium. *Cell* 1993;**75**(7):1305-15.
4. Jones AC, Shyamsundar MM, Thomas MW*, et al.* Comprehensive mutation analysis of TSC1 and TSC2-and phenotypic correlations in 150 families with tuberous sclerosis. *Am J Hum Genet* 1999;**64**(5):1305-15.
5. van Slegtenhorst M, Verhoef S, Tempelaars A*, et al.* Mutational spectrum of the TSC1 gene in a cohort of 225 tuberous sclerosis complex patients: no evidence for genotype-phenotype correlation. *J Med Genet* 1999;**36**(4):285-9.
6. Au KS, Rodriguez JA, Finch JL*, et al.* Germ-line mutational analysis of the TSC2 gene in 90 tuberous-sclerosis patients. *Am J Hum Genet* 1998;**62**(2):286-94.
7. Dabora SL, Jozwiak S, Franz DN*, et al.* Mutational analysis in a cohort of 224 tuberous sclerosis patients indicates increased severity of TSC2, compared with TSC1, disease in multiple organs. *Am J Hum Genet* 2001;**68**(1):64-80.
8. Sancak O, Nellist M, Goedbloed M*, et al.* Mutational analysis of the TSC1 and TSC2 genes in a diagnostic setting: genotype--phenotype correlations and comparison of diagnostic DNA techniques in Tuberous Sclerosis Complex. *Eur J Hum Genet* 2005;**13**(6):731-41.
9. Niida Y, Lawrence-Smith N, Banwell A*, et al.* Analysis of both TSC1 and TSC2 for germline mutations in 126 unrelated patients with tuberous sclerosis. *Hum Mutat* 1999;**14**(5):412-22.

10. Langkau N, Martin N, Brandt R*, et al.* TSC1 and TSC2 mutations in tuberous sclerosis, the associated phenotypes and a model to explain observed TSC1/ TSC2 frequency ratios. *Eur J Pediatr* 2002;**161**(7):393-402.
11. Cheadle JP, Reeve MP, Sampson JR*, et al.* Molecular genetic advances in tuberous sclerosis. *Hum Genet* 2000;**107**(2):97-114.
12. Henske EP, Scheithauer BW, Short MP*, et al.* Allelic loss is frequent in tuberous sclerosis kidney lesions but rare in brain lesions. *Am J Hum Genet* 1996;**59**(2):400-6.
13. Nellist M, van Slegtenhorst MA, Goedbloed M*, et al.* Characterization of the cytosolic tuberin-hamartin complex. Tuberin is a cytosolic chaperone for hamartin. *J Biol Chem* 1999;**274**(50):35647-52.
14. Garami A, Zwartkruis FJ, Nobukuni T*, et al.* Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. *Mol Cell* 2003;**11**(6):1457-66.
15. Nellist M, Sancak O, Goedbloed MA*, et al.* Distinct effects of single amino-acid changes to tuberin on the function of the tuberin-hamartin complex. *Eur J Hum Genet* 2005;**13**(1):59-68.
16. Roach ES, DiMario FJ, Kandt RS*, et al.* Tuberous Sclerosis Consensus Conference: recommendations for diagnostic evaluation. National Tuberous Sclerosis Association. *J Child Neurol* 1999;**14**(6):401-7.
17. Nellist M, Verhaaf B, Goedbloed MA*, et al.* TSC2 missense mutations inhibit tuberin phosphorylation and prevent formation of the tuberin-hamartin complex. *Hum Mol Genet* 2001;**10**(25):2889-98.
18. van Slegtenhorst M, Nellist M, Nagelkerken B*, et al.* Interaction between hamartin and tuberin, the TSC1 and TSC2 gene products. *Hum Mol Genet* 1998;**7**(6):1053-7.
19. Isaacs H, Jr. Fetal and neonatal cardiac tumors. *Pediatr Cardiol* 2004;**25**(3):252-73.
20. Maheshwar MM, Cheadle JP, Jones AC*, et al.* The GAP-related domain of tuberin, the product of the TSC2 gene, is a target for missense mutations in tuberous sclerosis. *Hum Mol Genet* 1997;**6**(11):1991-6.

APPENDIX

Preliminary data on the activation of the Hamartin-Tuberin Complex by AMPK Phosphorylation

INTRODUCTION

The major function of the hamartin-tuberin complex has been shown to be the inhibition of mTOR. The mTOR signaling pathway is one of the central pathways regulating cell size [1-3]. When activated, mTOR turns on protein synthesis by at least two mechanisms: 1- the phosphorylation of 4EBP-1 and the subsequent release and activation of translation initiation factor eIF4E, and 2- the phosphorylation and activation of S6K and, subsequently, of the 40S ribosomal protein S6. Tight regulation of this pathway by cellular energy and nutrient levels is essential since protein synthesis utilizes approximately 20-25% of the total cellular energy.

However, the biological basis of this regulation was not known until recently. It was first suggested that mTOR, through its high K_m for ATP, could function as a sensor for cellular ATP levels [4]. But since the ATP concentration is significantly higher than mTOR's K_m for ATP, drastic decreases in ATP levels are required to bring about changes in mTOR activity [5]. AMP, on the other hand, is a much better indicator of cellular energy levels because of its lower concentration. A relatively small decrease in ATP concentration will result in a relatively large increase in AMP levels [6] and therefore 5'-AMP-activated protein kinase (AMPK), which is sensitive to increased AMP levels, has been proposed as a physiological cellular energy regulator. AMPK has been shown to regulate mTOR signaling [7, 8], and tuberin was identified as the molecular link between AMPK and mTOR [9]. AMPK phosphorylates tuberin at two sites, amino acids T1271 and S1364, thereby activating it (see figure 5 on page 27).

Phosphorylation of tuberin by Akt, on the other hand, has an inhibitory effect through destabilization and/or disruption of the hamartin-tuberin complex. In addition to this direct effect, Akt also phosphorylates and inhibits AMPK, thereby reducing the activation of tuberin by AMPK [10]. The activating mechanism of AMPK phosphorylation on tuberin function remains to be elucidated and we started to investigate it.

EXPERIMENTAL WORK – PRELIMINARY RESULTS

As a first step to understand how hamartin-tuberin function is regulated by AMPK biochemically, we introduced the T1271A and S1364A mutations to the full-length wild-type tuberin construct. These tuberin variants are thought to be resistant to AMPK phosphorylation. Using our *in vitro* assays [11], the tuberin variants T1271A and S1364A and the variant carrying both mutations ("double A" variant) were found to retain rheb-GAP activity and were able to inhibit S6K and S6 phosphorylation (data not shown). Therefore, in contrast to previous reports [9, 12], our results

did not show that AMPK phosphorylation was necessary for tuberin function. However, it must be noted that our *in vitro* assays rely on protein overexpression and may lack the sensitivity to distinguish between the presence and absence of activation.

We did, however, observe an effect of the disruption of both the T1271 and S1364 phosphorylation sites. Using double-label immunofluorescent microscopy we found that hamartin staining was abnormal when hamartin was coexpressed with the tuberin “double A” variant (data not shown). Many cells exhibited a punctate hamartin staining pattern. Previously, we observed such hamartin staining in cells without exogenous coexpression of tuberin or coexpression of tuberin variants that are unable to form a complex with hamartin, e.g. the R611Q variant [11]. This observation suggests either that AMPK phosphorylation is necessary for complex formation, or that AMPK phosphorylation is important for the solubility/localization of the complex.

Next, we investigated the effect of 2-deoxyglucose (2-DG), a potent AMPK activator, and serum, an activator of mTOR signaling through growth hormone receptors. It was shown that, when cells expressing the wild-type hamartin-tuberin complex were treated with 2-DG, ~65% of them exhibited the punctate hamartin staining pattern compared to ~25% of those treated with serum (figure 1.) On the other hand, approximately 50% of the cells expressing the T1271A, S1364A, “double A” or R611Q variants, exhibited a punctate pattern, irrespective of the treatment. This suggests that the difference in the staining pattern of wild-type complex using 2-DG and serum is dependent on the phosphorylation of tuberin on both sites by AMPK.

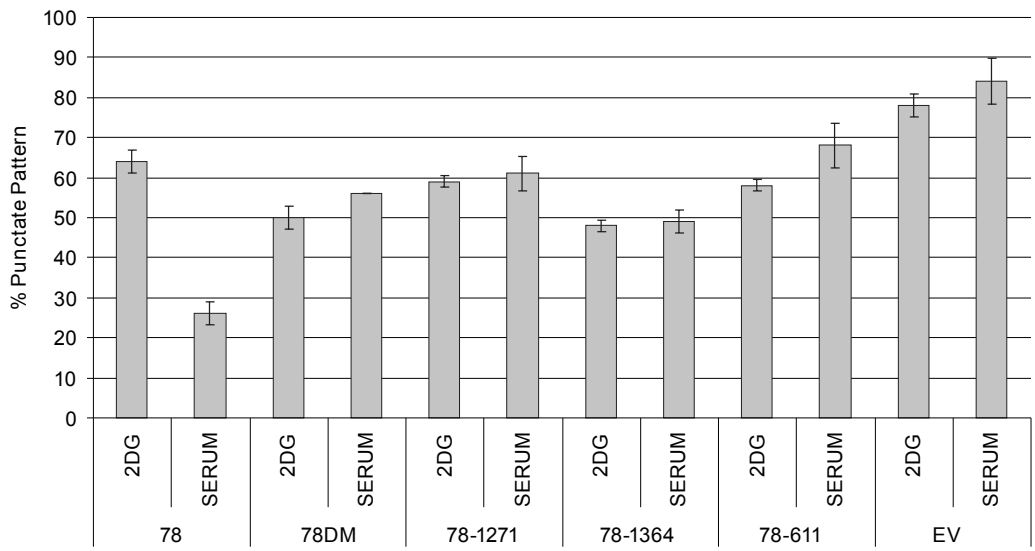


Figure 1: The effects of AMPK phosphorylation on hamartin staining. Cells expressing wild-type hamartin-tuberin (78) complex when treated with 2-DG exhibit 65% punctate hamartin staining pattern, while when treated with serum exhibit only 25% punctate hamartin staining pattern. Cells expressing hamartin with tuberin variants resistant to AMPK phosphorylation or with the R611Q variant, irrespective of treatment, show 50% punctate hamartin staining pattern. Empty vector (EV) with hamartin was used as a negative control.

Subsequently, we used a co-immunoprecipitation assay to determine whether the abnormal staining pattern was due to disrupted hamartin-tuberin complex. Using antibodies against hamartin, we were able to immunoprecipitate wild-type tuberin as well as the T1271A, S1364A and “double A” tuberin variants. This strongly suggests that the hamartin-tuberin interaction does not require phosphorylation of tuberin by AMPK. In other words, AMPK phosphorylation does not seem to enhance hamartin-tuberin complex formation, but presumably activates the complex after its formation.

In order to explore whether AMPK phosphorylation affects the solubility of the complex, we performed fractionation experiments. In these experiments, we showed that the ratio of insoluble to soluble hamartin-tuberin complex was increased for the wild-type tuberin when cells were treated with the AMPK activator 2-DG. On the other hand, addition of 2-DG had no effect on the ratio of insoluble to soluble complex for the tuberin variants T1271A, S1364A or “double A”. This suggests that AMPK phosphorylation helps to relocate - or change the solubility of - the hamartin-tuberin complex.

PRELIMINARY CONCLUSIONS AND FUTURE DIRECTIONS

Protein synthesis is a vital cellular process that needs to be tightly coordinated with various other cellular processes and physiological conditions. Inhibition of translation is a major physiological response under energy starvation conditions to maintain cellular homeostasis. Yet, the biochemical mechanism of this response is poorly understood. Recent research has placed AMPK in the mTOR signaling pathway, upstream of the hamartin-tuberin complex. The complex is activated by AMPK under energy starvation conditions, and consequently inhibits the mTOR signaling pathway. However, the precise biochemical mechanism of how AMPK phosphorylation leads to complex activation is not yet known.

Our results suggest that the phosphorylation of tuberin by AMPK does not have an effect on hamartin-tuberin complex formation, but it seems to facilitate the migration of the already formed complex to the membrane. It may be of relevance that rheb, the molecular target of the tuberin GAP activity, is located on the membrane [13].

If phosphorylation by AMPK does have an effect on migration of the complex, tuberin variants that behave as if they were phosphorylated by AMPK are expected to be predominantly present in the insoluble fraction and cells expressing these tuberin variants along with hamartin are expected to exhibit a predominantly punctate hamartin staining pattern, irrespective of treatment. We introduced the phosphomimetic T1271D and S1364D mutations into the full-length wild-type tuberin construct to investigate their effects. Another interesting step in this line of research would be to investigate the effect of AMPK activation on green fluorescent protein (GFP)-labeled tuberin or hamartin localization. The main advantage of using such a tag is the possibility of performing experiments in live cells. Other proteins regulating the hamartin-tuberin complex could also function by relocating the complex to or away from its cellular site of action.

Further studies of the hamartin-tuberin complex and its regulation will result in a better understanding of the pathogenesis of various lesions and developmental abnormalities observed in TSC and many other disorders, and will yield better therapeutic options.

REFERENCES

1. Potter, C.J., L.G. Pedraza, and T. Xu, *Akt regulates growth by directly phosphorylating Tsc2.* Nat Cell Biol, 2002. **4**(9): p. 658-65.
2. Gao, X. and D. Pan, *TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth.* Genes Dev, 2001. **15**(11): p. 1383-92.
3. Garami, A., et al., *Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2.* Mol Cell, 2003. **11**(6): p. 1457-66.
4. Dennis, P.B., et al., *Mammalian TOR: a homeostatic ATP sensor.* Science, 2001. **294**(5544): p. 1102-5.
5. Proud, C.G., *Control of the translational machinery in mammalian cells.* Eur J Biochem, 2002. **269**(22): p. 5337.
6. Hardie, D.G., D. Carling, and M. Carlson, *The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell?* Annu Rev Biochem, 1998. **67**: p. 821-55.
7. Kimura, N., et al., *A possible linkage between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signalling pathway.* Genes Cells, 2003. **8**(1): p. 65-79.
8. Krause, U., L. Bertrand, and L. Hue, *Control of p70 ribosomal protein S6 kinase and acetyl-CoA carboxylase by AMP-activated protein kinase and protein phosphatases in isolated hepatocytes.* Eur J Biochem, 2002. **269**(15): p. 3751-9.
9. Inoki, K., T. Zhu, and K.L. Guan, *TSC2 mediates cellular energy response to control cell growth and survival.* Cell, 2003. **115**(5): p. 577-90.
10. Hahn-Windgassen, A., et al., *Akt activates mTOR by regulating cellular ATP and AMPK activity.* J Biol Chem, 2005.
11. Nellist, M., et al., *Distinct effects of single amino-acid changes to tuberin on the function of the tuberin-hamartin complex.* Eur J Hum Genet, 2005. **13**(1): p. 59-68.
12. Shaw, R.J., et al., *The LKB1 tumor suppressor negatively regulates mTOR signaling.* Cancer Cell, 2004. **6**(1): p. 91-9.
13. Yu, Y., et al., *Structural basis for the unique biological function of small GTPase RHEB.* J Biol Chem, 2005. **280**(17): p. 17093-100.

3

DISCUSSION

3. DISCUSSION

3.a. Mutation Analysis

Mutation Detection Rates

The identification and characterization of the responsible genes, the *TSC1* and *TSC2* genes, enabled the determination of the mutation spectra of the *TSC1* and *TSC2* genes [1-10]. Mutations were found more frequently at the *TSC2* locus. In *de novo* cases *TSC2* mutations are 2-10 times more frequent [6-8, 10, 11]. In familial cases, the numbers are closer to each other, but *TSC2* mutations are still more common [7, 8, 10].

The size, complexity of structure and chromosomal location of a gene all play roles in whether a locus is more prone to replicative errors. The average number of mutations per nucleotide for *TSC2* gene is more than two times higher than that for *TSC1* [10]. This simple observation indicates that the difference in the mutation frequencies of the two genes is not only a matter of gene size. The lower number of mutations in the *TSC1* gene is also partly related to the reported absence or the very low frequency of large DNA rearrangements, missense and splice site mutations. Such replicative errors in the *TSC1* gene do not happen frequently, do not result in protein dysfunction, or may have an embryonic lethal effect. The resulting phenotype in case of no or mild effect on protein function could be missed using the current clinical diagnostic criteria.

For a comprehensive mutation analysis of the *TSC1* and *TSC2* genes, it is necessary to use a combination of methods to cover the detection of small nucleotide changes as well as larger rearrangements. For the detection of small nucleotide changes in our large cohort, we used DGGE because of its high sensitivity and high throughput. For exons for which DGGE was not possible due to technical difficulties, we used either SSCP or direct sequencing. SSCP had been in use for mutation screening since the identification of the *TSC1* and *TSC2* genes in our laboratory and therefore some samples were screened by both DGGE and SSCP. For the detection of larger rearrangements affecting more than one exon of the *TSC2* gene, we used FISH and Southern blotting.

Different laboratories used different combinations of techniques and those reporting on larger cohorts consistently have a mutation detection range of 80-90% in patients with definite TSC [6, 7, 10]. There may be several reasons why we are unable to identify mutations in 10-20% of the patients.

Missed Mutations?

Technical difficulties related to mutation analysis, medium size deletion mutations missed by both PCR-based and probe-related screening techniques such as long-range PCR, FISH and Southern analysis, mutations within the promoter/enhancer or intronic regions, and somatic mosaicism probably all contribute to mutation detection failure [12]. For the detection of small nucleotide changes we used a combination of DGGE, SSCP and direct sequencing on our large cohort [10]. In patients with known mutations and where both SSCP and DGGE were performed, we have shown

a higher sensitivity of DGGE (84% vs. 93% respectively). However, none of the techniques has a sensitivity of 100%.

In contrast to other studies, Longa et. al. reported three large deletions in the *TSC1* gene in a cohort of 202 patients, accounting for 1.5% of all the mutations identified in the *TSC1* and *TSC2* genes [13]. The same group continued their efforts searching for large rearrangements, and identified seven additional deletions involving one or more exons of the *TSC1* gene, four of which also included the promoter region (Nicola Migone, personal communication). According to their results, the ratios of large deletions to the total number of mutations in both genes are similar, approximately 10%. We have identified and characterized such a large deletion in the *TSC1* gene after the coincidental finding of an informative intragenic polymorphism that showed an inheritance pattern consistent with the presence of a deletion in the affected individuals in the family (Chapter 2.b). Since the *TSC1* gene is not screened for large rearrangements in routine diagnostic mutation analysis, several of these mutations may have been missed.

For the detection of larger rearrangements affecting more than one exon of the *TSC2* gene, we used a combination of FISH and Southern blotting. In only 50% of the cases with larger rearrangements both techniques were able to detect the abnormality. At the moment a new technique, the Multiplex Ligation-dependent Probe Amplification (MLPA), is being evaluated for the detection of medium-large deletion mutations. This relatively new technique is a fast and efficient PCR-based system to determine the copy number of up to 45 different sequences of interest. However, since polymorphisms or single base mutations in the probe binding regions may affect MLPA results, single base changes may appear as exon deletions. For this reason, all simple fragment deletions indicated by MLPA should be confirmed by an independent method [14]. MLPA can also yield inconclusive results when performed on old and low-quality DNA samples. In our laboratory, MLPA on 119 informative samples that had been prescreened for small mutations yielded 21 aberrant patterns. Two of these have been confirmed to be large deletions using FISH analysis, one encompassing *TSC2* exons 21-41 and the other one a whole *TSC2* gene deletion (van den Ouweland, unpublished data). At the moment, new FISH probes are being evaluated for the confirmation of the deletions identified in the N-terminal part of the *TSC2* gene by MLPA.

Splicing abnormalities due to sequence changes distant from exon-intron boundaries seem to happen infrequently in *TSC* genes. However, Mayer et al identified eight aberrant polypeptides (one in the *TSC1* and seven in the *TSC2* gene) using an RNA-based protein truncation test (PTT) in 48 patients prescreened for large intragenic *TSC2* rearrangements [11]. The causative mutations for the aberrant polypeptides could not be determined. These might lay deep within introns or might as well be false positives related to the PTT technique. The 28 mutations these authors did identify were either truncating mutations in the coding region or mutations affecting splicing, all but one would also have been detected by genomic screening techniques on exons and exon-intron boundaries [15]. Mutations in the enhancer/promoter regions also seem to be infrequent. Large deletions however, frequently include the promoter as well (4 of 10 *TSC1* deletions and 19 of 33 *TSC2* deletions) (Nicola Migone, personal communication).

Somatic mosaicism, on the other hand, as in other syndromes related to tumor suppressor genes [16, 17], is well documented in TSC in both probands and parents of TSC patients [18-23]. In a recent study, somatic mosaicism was identified in 3% of the patients diagnosed with definite TSC [19]. In our cohort, we have detected only one case of somatic mosaicism in an index case [10]. The patient had retinal phakomas and depigmentations, subependymal nodules, kidney cysts, facial angiofibromas, hypomelanotic macules, seizures and borderline mental retardation. The deletion was present in 80% of the leukocytes investigated, which suggests high-grade mosaicism, although no other tissues were available for examination. Kwiatkowska et al illustrated that a patient with 30% somatic mosaicism can also be severely affected [23]. This patient had epilepsy, multiple SENs and tubers, severe mental retardation and facial angiofibromas, whereas the results of renal ultrasonography, echocardiography, and retinal examination were all normal. Jones et al described somatic mosaicism in two index cases who were mildly affected and in one parent who had only minor skin signs of TSC [22]. The mutations involved escaped detection by regular sequencing and could be identified at low frequency (6-17%) after cloning of the appropriate amplicons. In a previous study, our group showed somatic mosaicism in approximately 10% of the parents (6/62) and these parents were diagnosed after identification of the mutation in the proband's DNA, had normal intelligence and no history of epilepsy. In addition to six previously reported parental somatic mosaicism cases and the aforementioned somatic mosaicism in an index case, we identified somatic mosaicism in one more parent [10]. Since the use of labor-intensive allele specific methods is no longer included in our diagnostic routine, it is possible that we were unable to identify all the somatic mosaic parents in our latest study. However, a recent study by Roberts et al showed that somatic mosaicism is rare in unaffected parents of TSC patients [19]. The debate whether somatic mosaic mutations are associated with milder phenotypes will require clinical investigation of more TSC patients with such mutations and quantification of the degree of mosaicism in these patients. However, mathematically the hypothesis seems probable. If the number of cells carrying the first mutation is lower, the chance that those cells will acquire a second hit will also be lower, thus limiting the number of possible TSC associated lesions. The patients in our cohort with no identified mutations had milder phenotypes. At least a proportion of these patients could be somatic mosaics, since it is technically difficult to show low-level somatic mosaicism. Moreover, the available DNA was from leukocytes or cultured fibroblasts and mosaicism that was limited to specific organs and tissues would escape detection altogether (see Chapter 3.b.).

Or a Third Locus?

Does a third locus exist for TSC? This question has been asked since the first half of the 1990s. Prior to the identification of the chromosome 16 locus, linkage analysis revealed loci on chromosomes 11 and 12 in addition to the chromosome 9 locus but the linkage was weak and not reproducible [24-26]. Current evidence does not support an additional gene that might cause TSC. However, the main functions of the protein products of the *TSC1* and *TSC2* genes seem to be related to the signaling through the mTOR pathway and this pathway involves many other proteins (see Chapter 3.e.).

After the identification of rheb as the direct target of the tuberin GAP activity, it was suggested that

mutations in *rheb* could result in phenotypes similar to that of TSC [27, 28]. In 24 index cases with a definite clinical diagnosis of TSC where we were unable to identify a pathogenic mutation in either the *TSC1* or the *TSC2* gene, we screened the *rheb* gene by SSCP and direct sequencing (unpublished data). We identified 4 previously unreported nucleotide changes in the intronic sequences of the rheb gene, IVS1+60C>T, IVS5-7G>A, IVS6+59del3 and IVS7-35T>C. Splice site prediction analysis indicated that none of the changes were likely to have an effect on splicing and were therefore unlikely to be responsible for TSC in these patients. We concluded that *rheb* is not a good candidate disease gene for TSC. However, as of yet, the guanine nucleotide exchange factor(s) for rheb is not identified. Even if mutations in genes encoding such proteins would be unlikely to cause TSC, the activity of these could play a role in the phenotypic expression of the disease.

The question then becomes “Could specific mutations in other genes regulating the same pathway result in similar phenotypes?”. To answer this question one has to look at the phenotypes resulting from mutations in these genes and have a good understanding of the functions of the network of pathways involved.

There are several disorders characterized by familial hamartomas. Peutz-Jeghers syndrome (PJS) in particular, has striking resemblances to TSC [29, 30]. Patients with PJS have predominantly gastrointestinal hamartomatous polyps, however histopathologically these hamartomas are strikingly similar to hamartomas seen in TSC. This syndrome is caused by mutations in the *LKB1* (also known as *STK11*) gene. The protein product of the gene is a serine/threonine kinase, the best-known substrate of which is AMPK, which in turn phosphorylates and activates tuberin. Activation of the mTOR signaling pathway has been shown in cell biological systems as well as hamartomatous polyps from mouse models of PJS [31, 32].

Activation of the mTOR signaling pathway has also been shown for hamartomatous syndromes related to the tumor suppressor gene *PTEN* (Cowden disease, Bannayan-Riley-Ruvalcaba syndrome, Lhermitte-Duclos disease and Proteus syndrome) [33-37]. PTEN is a phosphatase specific for 3’ phosphoinositides and loss of PTEN leads to an increase in membrane concentrations of these chemicals. Among other effects, this leads to increased activation of AKT, which in turn phosphorylates and negatively regulates tuberin.

In short, dysregulation of the mTOR signaling pathway seems to play a central role in hamartomatous tumor growth [30]. Both LKB1 and PTEN are involved in regulating other pathways, which may in part help explain the phenotypic differences and tissue distribution seen in these disorders compared to TSC [38, 39]. However, specific mutations in these genes, involving specific amino acids that are only necessary for interacting with the mTOR signaling pathway could theoretically result in a phenotype similar to TSC. Other genes involved in the pathway may be candidates as well. For example a mutation in the gene encoding AMPK that causes only a failure to activate tuberin or that hinders the AMPK-tuberin interaction might have a similar effect as a missense mutation in *TSC2* resulting in tuberin with some residual activity.

Rare Polymorphism or Pathogenic Mutation?

Not every abnormal pattern, nucleotide change or small deletion identified using mutation analysis is necessarily a pathogenic mutation [10](Chapter 2.e). The utilization of mutation analysis as a diagnostic and counseling tool requires a rigorous protocol to distinguish between rare polymorphisms and pathogenic mutations.

A sequence change found can be an already known pathogenic mutation or polymorphism (see figure 1). Several recurrent mutations and common polymorphisms have been published for both the *TSC1* and *TSC2* genes. Although not updated, a database with mutations and polymorphisms accompanied by the references and the supporting evidence is available on the internet (http://tsc-project.partners.org/index.htm). If the aberration identified is previously unpublished, and is causing a shift in the reading frame (frameshift), or a premature stop codon (nonsense) or is a large rearrangement (duplication, inversion, large deletion, translocation) the aberration is designated a disease-causing mutation. However, if the identified aberration is an amino acid substitution, a nucleotide change possibly affecting splicing or an in-frame nucleotide change (deletion or insertion of nucleotides in an order of 3), the pathogenicity of the aberration has to be established. Since two-thirds of the cases have a *de novo* mutation, the first step in determining the nature of such an aberration is to analyze parents of the affected individual. In the mean time, as much clinical data as possible is gathered about the family. If the aberration is *de novo*, with clinically unaffected parents, it is accepted as the pathogenic mutation for the individual. If the aberration is not *de novo* however, the change is classified as an unknown nucleotide change.

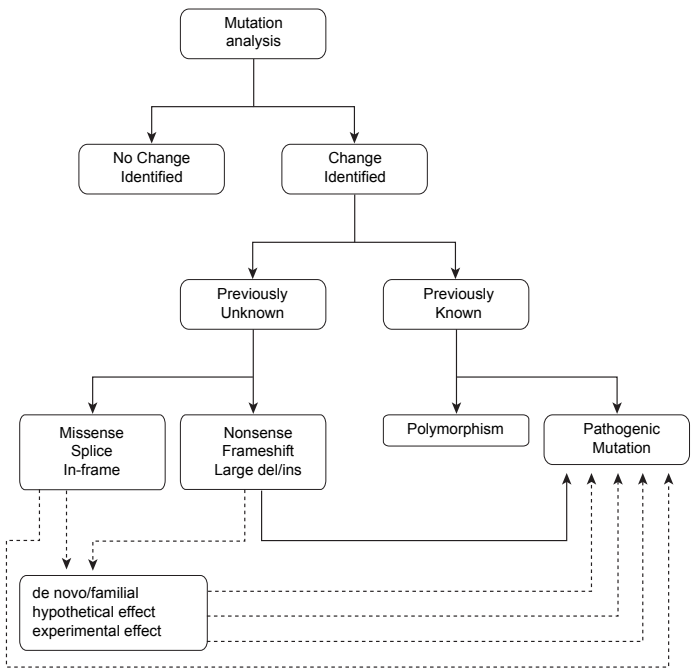


Figure 1: Decision making in mutation analysis. A sequence change identified could be a previously known polymorphism or pathogenic mutation. If the identified sequence change is not previously published though, and is causing a premature stop or frameshift, the change is accepted as a disease causing mutation. For large rearrangements it is preferable to show the breakpoints, whether the rearrangement leads to frameshift and whether it is *de novo*. Splice-site changes that involve the conserved nucleotides in splice-sites (-1, -2, +1 and +2 positions) are accepted as pathogenic mutations. For missense, other splice-site and in-frame sequence changes, first parental analysis is performed and *de novo* changes are accepted as pathogenic mutations. If not, *in silico* analysis is performed followed by biochemical assays where indicated and possible.

We analyze the unknown nucleotide changes with 3 different splice site analysis programs (www.fruitfly.org/seq_tools/splice.html; www.cbs.dtu.dk/services/NetGene2 and www.genet.sickkids.on.ca/~ali/splicesitefinder.html). In addition, for missense changes, we calculate the three amino acid substitution matrix scores and investigate whether the amino acid in question is evolutionarily conserved (see Chapter 3.b). If there are no theoretical effects using these computational analyses, the nucleotide change cannot be classified as either a pathogenic mutation or a rare polymorphism. Even when the parent carrying the nucleotide change is clinically disease-free, because of the highly variable phenotypic expression of TSC, the nucleotide change must still be considered as a potentially pathogenic mutation. Conversely, even if the parent carrying the nucleotide change is a symptomatic TSC patient, the nucleotide change can simply be a rare polymorphism cosegregating with the disease. If a hypothetical effect is found, on the other hand, the effect needs to be proven before diagnostic application is considered. For an effect on splicing, mRNA from cultured fibroblasts of the affected individual is isolated and tested for aberrant splice forms. For missense and in-frame changes, the nucleotide change is cloned into a mammalian expression vector, and the effects of the change on protein function using biochemical assays are tested (see Chapter 3.b).

3.b. Functional Analysis

In Silico Analysis

Making the distinction between (rare) polymorphisms and pathogenic mutations, especially for non-truncating nucleotide changes, can be very difficult. We analyze the segregation of such nucleotide changes found in the *TSC2* gene in families since a *de novo* change in the absence of another possible pathogenic mutation is highly indicative of a pathogenic mutation. Where this information is not available (e.g. where key relatives are not accessible), or where the nucleotide change co-segregates with TSC in the family, the distinction between a polymorphism and a pathogenic mutation cannot be made on genetic grounds only.

For such cases, evolutionary conservation of the amino acid in different species is investigated in addition to evaluating if amino acid substitutions might have functional consequences using amino acid substitution matrices. Evolutionary conservation of an amino acid is a good indication of whether an amino acid is located in a functionally important domain and whether it is important for the function of the protein. Log-odds amino acid substitution matrices, which are point/percent accepted mutation matrix (PAM) and block substitution matrix (BLOSUM), are based on the observed frequencies of amino acid substitutions in alignments of related proteins. PAM matrix is calculated by observing the differences in closely related proteins. PAM1 matrix measures the substitution rates when 1% of the amino acids have changed and PAM250 matrix is calculated by extrapolation of this data. Theoretical scores for PAM250 matrix are between –8 and 17. BLOSUM series of matrices are calculated using homologous regions of multiple alignments of evolutionarily divergent proteins. The most frequently used BLOSUM62 matrix includes only blocks of sections of proteins that share at least 62% sequence identity. Theoretical scores for BLOSUM62 are between –4 and 11. The higher the value for PAM250 and BLOSUM62 matrices, the more likely a substitution is found in natural proteins, and the less likely such a substitution is a pathogenic

mutation. Grantham matrix on the other hand, uses chemical properties of amino acid side chains (composition, polarity and molecular volume) to calculate the overall difference between any two amino acids. For this matrix, theoretical scores are between 5 and 205, and the higher the Grantham scores, the less similar the amino acids compared and hence the more likely that the amino acid substitution has functional consequences. Amino acid substitution matrices indicate that arginine to glutamine change (R>Q) is a conservative change and unlikely to have functional implications (PAM250 score=1, BLOSUM62 score=1, Grantham score=43) for example. However, this amino acid substitution is found both as pathogenic mutation and as polymorphism along the *TSC2* gene. Interestingly, the arginines at positions 367 and 917 are evolutionarily conserved in vertebrates, but R>Q substitutions at these positions are polymorphisms. However, arginines at positions 611, 905 and 1720 are evolutionarily conserved in vertebrates as well as in *Drosophila melanogaster* and *Anopheles gambiae*, and R>Q substitutions at these positions are pathogenic mutations. Although amino acid substitution matrices give an idea whether two amino acids are chemically similar and/or conserved, and evolutionary conservation of amino acids give an idea whether an amino acid is indispensable for the function of the protein, it must be noted that the same amino acid substitution might have different consequences depending on the surrounding amino acids and the domain it is located at (Chapter 2.e).

Biochemical Analysis

In selected cases with non-truncating nucleotide changes in the *TSC2* gene, where the family members are available and/or where the *in silico* analyses indicate a possible effect, we perform biochemical assays in order to determine whether such a change has functional implications. In addition, biochemical assays make it possible to determine what kind of functional consequences a mutation has and they allow the effects of different mutations to be compared. For these purposes, missense or in-frame nucleotide changes are introduced into a mammalian expression vector and these constructs are transfected into various cell types for different types of functional assays.

The biochemical assays we use address:

1- Complex formation
2- Rheb-GAP activity of the complex
3- Inhibition of S6K phosphorylation
4- Inhibition of S6 phosphorylation

The results of biochemical assays should be carefully interpreted because of limiting factors and (dis-)advantages related to each assay. Since the phenotypes associated with mutations in the *TSC1* and *TSC2* genes are so similar, it was hypothesized and shown that hamartin and tuberin bind to each other and form a functional complex in vivo [40, 41]. Amino acid substitutions that do not cause TSC (polymorphisms) do not decrease hamartin-tuberin binding [42](Chapter 2.d and 2.e). On the other hand, the majority of the studied disease-causing missense mutations in the *TSC2* gene have been shown to disrupt this interaction. Therefore, amino acid substitutions and deletion/insertion of amino acids that disrupt hamartin-tuberin interaction are highly likely to be pathogenic mutations, but the result of the binding assay alone is not conclusive.

The three other assays are to test whether the mutation in *TSC2* gene decreases the efficiency of tuberin in inhibiting the mTOR signaling pathway, in other words, whether the mTOR pathway is activated in the presence of the mutant tuberin variant. Activation of the mTOR signaling pathway has been shown in various cell types in TSC associated lesions, including giant cells of tubers, renal angiomyolipomas and cardiac rhabdomyomas. Tuberin regulates mTOR and the downstream effectors of mTOR via its GAP activity towards rheb. To measure this activity, immunoprecipitated complex is incubated with rheb preloaded with radioactively labeled GTP and subsequently the GDP/GTP ratio is measured. It must be noted, however, that the GAP activity measured will be substantially lower when the complex cannot be formed, or substantially higher if endogenous complex from the transfected cells is co-precipitated.

Inhibition of S6K phosphorylation is a semi-quantitative assay where *S6K* along with *TSC1* and *TSC2* is transfected into cells. For this purpose, we use 293 human embryonal kidney (HEK) cells in which the expression of endogenous S6K is negligible. The expression of the endogenous TSC1 and TSC2 on the other hand should always be kept in mind. Under certain circumstances (prolonged serum starvation, addition of AMPK activators or inhibitors of Akt) the activity of the endogenous complex could be sufficient to inhibit phosphorylation of the exogenous S6K up to a level. Therefore, the results of such experiments should be interpreted using proper internal positive and negative controls for each experiment.

The assay to investigate the inhibition of S6 phosphorylation is a semi-quantitative method, performed on *Tsc2* -/- mouse embryo fibroblasts (MEFs) in which S6 is constitutively phosphorylated. In this case, *TSC1* and *TSC2* are cotransfected into MEFs and the phosphorylation status of S6 is investigated under fluorescence microscopy in transfected cells exclusively, minimizing the background.

In order to use functional assays to determine whether a nucleotide change identified in mutation analysis is pathogenic or not, the results of the functional analysis have to be reproducible and inter-experimental differences have to be minimal. Clinical, genetic and functional analysis data should be combined and carefully reviewed.

Functional assays also provide a means of comparing the effects of different pathogenic mutations in the *TSC2* gene. While some mutations disrupt hamartin-tuberin interaction and show deleterious effects in the other assays (e.g. the R611Q mutation) [43], others may affect tuberin GAP activity only (e.g. the L1511H mutation) (Chapter 2.e). There might yet be other mutations, which only prevent AMPK activation of the complex (see Chapter 2 appendix). Studying different non-truncating mutations located along the *TSC2* gene will help identify domains and amino acids important for different aspects of the hamartin-tuberin complex function.

Functional Analysis in Practice

Functional assays complement the existing diagnostic tests and enable identification and characterization of pathogenic mutations in difficult cases. However, functional assays are very

time-consuming and expensive for routine use. Therefore, at present, the use of functional analysis is restricted.

We performed functional assays for over 20 non-truncating nucleotide changes in the *TSC2* gene. We were able to show the dysregulation of the mTOR signaling pathway for pathogenic mutations and at present the results of various functional assays for each pathogenic mutation tested have been in agreement with each other.

In order to speed up the process of testing possible pathogenic mutations, a high throughput functional assay is being developed in our laboratory. S6K phosphorylation status has been shown to be the simplest and most reliable assay for hamartin-tuberin function by our group and others [43, 44]. This new approach will make use of co-expression of S6K, hamartin and tuberin variants in 293 cells in multi-well culture plates and will enable simultaneous testing of multiple variants. This will reduce the time required for the functional assays and the costs significantly.

Other Possible Assays for the Functions of the Hamartin-Tuberin Complex

For diagnostic purposes, hamartin-tuberin complex formation and the main pathway involved in the pathogenesis of the TSC associated lesions, which is the mTOR signaling pathway, are investigated in our laboratory. However, for a better understanding of the functions of the hamartin-tuberin complex and in which ways non-truncating mutations disrupt these functions, there are a variety of biochemical assays that can be applied now or in the future.

We have shown that various tuberin variants were not detected by the antibody specific for the recognition site of the Akt kinase [43]. In addition, while expression of the active Akt isoform resulted in the appearance of low mobility isoforms of the wild-type tuberin and tuberin variants that were recognized by the Akt-substrate antibody, it resulted in no change in mobility for various tuberin variants. It must be noted however, that the tuberin variants that were not recognized by the antibody and that did not demonstrate a mobility shift in the presence of an active Akt isoform, were unable to form a complex with hamartin and unable to prevent activation of the mTOR signaling pathway. This suggests that such amino acid substitutions disrupt the correct conformation of the protein. Since phosphorylation of tuberin by Akt has an inhibitory effect on the function of the complex, an amino acid variation that only prevents phosphorylation of tuberin by Akt would not be expected to disrupt the inhibitory function of the complex on mTOR signaling pathway.

It has recently been shown that AMPK directly phosphorylates tuberin. This phosphorylation activates the hamartin-tuberin complex and in turn the complex inhibits signaling through mTOR. Specific antibodies can be used to determine which tuberin variants can be recognized by AMPK and phosphorylated. Amino acid substitutions that only prevent this activation without further interfering with the functions of the hamartin-tuberin complex could result in less drastic functional loss and may lead to a milder phenotype in patients (Chapter 3.c).

Other possible functional assays might include a binding assay for tuberin-rheb interaction, a

phosphorylation assay for 4EBP-1, a stability assay of the hamartin-tuberin complex, and assays measuring cell size. As we learn more about the functions of the hamartin-tuberin complex, there will be a growing list of assays applicable to TSC variants, which might possibly affect different aspects of their functions.

3.c. Phenotypic Expression of TSC

Development of TSC Associated Lesions

The *TSC1* and *TSC2* genes are considered to be tumor suppressor genes. In accordance with this, second hit events have been demonstrated in many lesions associated with TSC, e.g. renal AMLs, cardiac rhabdomyomas and SEGAs [45-53]. However, at present it is still uncertain whether all TSC associated lesions are caused by second hit events. It may be difficult to detect the presence of second hits in some lesions. In cortical tubers for example, activation of the mTOR signaling pathway has been demonstrated in giant cells, but it was not rigorously proven that this was due to a second hit [54, 55]. In some lesions, other pathogenic mechanisms may be at work. Phosphorylation of tuberin has been suggested as a pathogenic mechanism in tubers [56, 57]. Another possibility for the development of lesions is haploinsufficiency. At critical developmental stages, one normal copy of *TSC1* or *TSC2* may be insufficient for the normal functioning of the cell.

TSC1 vs. TSC2

Whether there was a difference between the phenotypes associated with mutations in the *TSC1* gene and those associated with mutations in the *TSC2* gene has been a question studied by many researchers over the years. Although the differences in the first couple of studies were not concrete, larger cohorts were able to show that patients with mutations in the *TSC2* gene were more likely to have a higher number and/or severity of clinical features compared to those with mutations in the *TSC1* gene [6, 7]. In this thesis, the largest TSC cohort reported to date is presented [10]. Compared to previous studies, we have shown a similar distribution of mutations along the *TSC1* and *TSC2* genes and similar spectra of mutations. Mutations in the *TSC2* gene were predominant both in *de novo* and in familial cases and these led to more frequent and more severe clinical manifestations. However, the spectrum of lesions and symptoms associated with mutations in the *TSC1* and *TSC2* genes are similar and there are no clinical manifestations specifically associated with either of the genes.

There are several hypotheses why *TSC1* mutations may result in less severe phenotypes compared to *TSC2* mutations. First, it is possible that second-hit events occur less often in the *TSC1* gene than in the *TSC2* gene. The lower number of lesions seen in patients with *TSC1* germline mutations implies a lower frequency of somatic mutations in the *TSC1* gene compared to *TSC2*, in parallel with the observed lower mutation frequency of germline mutations.

Another possibility is that the effects of complete loss of hamartin and tuberin are different. If tuberin and/or hamartin, besides forming a functional complex with each other, have independent roles in separate cellular processes in different tissues, loss of one or the other might have different

consequences. Tuberin, for example might have a yet to be discovered function in brain development independent of hamartin, and for this reason, loss of tuberin may result in more severe mental retardation. Conversely, hamartin might have an important role for cell survival, and loss of hamartin might result in cell death or early embryonic lethality. The main function of the hamartin-tuberin complex, however, seems to be regulation of the mTOR signaling pathway [30]. Tuberin via its GAP-domain seems to be the functionally active participant of the complex. Although the function of hamartin is not completely understood, current evidence indicates either a regulating or a chaperone role on tuberin function. In vitro assays demonstrate that in the absence of hamartin, tuberin has GAP activity towards rheb and can inhibit, although less efficiently, S6 phosphorylation (unpublished data). It is possible that loss of hamartin, in vivo, may also have less drastic consequences.

TSC2 Mutations with Milder Phenotypic Effects

Although in general more severe phenotypes are associated with mutations in the *TSC2* gene, some specific mutations in the *TSC2* gene are associated with milder phenotypes [58, 59] (Chapter 2.d and 2.e). Almost all the mutations identified in the *TSC1* gene are truncating mutations. Conversely, approximately 20% of the mutations identified in the *TSC2* gene are missense mutations and 7% are in-frame mutations [10] (Chapter 1.d). We have shown that some of these changes are inactivating mutations [43]. However, some mutations do not seem to cause a complete loss of tuberin function in vitro [43] (Chapter 2.d). In two families, where two non-truncating nucleotide changes were identified in each family, we used functional assays to determine whether any of the nucleotide changes was pathogenic. In one of these families, the index case, who had deceased postpartum, was diagnosed with a solitary cardiac rhabdomyoma without any further signs of TSC. The functional assays indicated that the pathogenic mutation (2476delATC in *TSC2* gene resulting in the deletion of isoleucine at position 820) was inherited from the father who had one nail groove and one hypomelanotic macule, which was insufficient for a clinical diagnosis of TSC. In the other family, the index case had hypomelanotic macules, multiple dental pits and epilepsy, and her half sister had cortical tubers, hypomelanotic macules and epilepsy. Both girls inherited the pathogenic mutation (4550T>A in the *TSC2* gene resulting in L1511H) from their father, who had only a 2-cm kidney cyst and an area of ash leaf-shaped hypopigmentation (Chapter 2.e) and did not meet the diagnostic criteria for TSC. Functional analyses revealed in the latter case that the mutant tuberin was able to form a complex with hamartin, which indicated that at least some tuberin function is preserved. In an extended family, the missense mutation 2714G>A in the *TSC2* gene, resulting in the R905Q amino acid substitution was identified in 25 mildly affected individuals (Chapter 2.d). In this particular family, diagnostic criteria for TSC were met in only a minority of the cases. We have shown that, compared to other mutations, the R905Q mutation had a milder effect on tuberin function [43] (Chapter 2.d). In short, there are mutations in the *TSC2* gene that have milder effects on tuberin function. In cells of the patients with such mutations, also after a second hit event, the residual activity of the tuberin variant containing the primary mutation might result in a milder phenotype. The association of particular mutations with mild phenotypes might be useful in genetic counseling of families with such mutations.

Phenotypic Differences Between Patients with and without Mutations

Another important observation was the reproducible and significant phenotypic differences between patients in whom no mutation could be identified and those with identified mutations [7, 10]. In our cohort, the group of patients with no identified mutations had lower frequencies in 15 of the 17 investigated clinical features of TSC, compared to those with an identified mutation. The differences were statistically significant for mental retardation, seizures, shagreen patches, hypomelanotic macules and forehead plaques. Failure of mutation detection in at least a portion of these patients may be attributed to somatic mosaicism. Many lesions associated with TSC have been shown to have lost the wild-type allele in addition to inherited or *de novo* germline mutation in the other allele. Depending on the developmental stage when the mutation arises, some tissues and organ systems might be completely spared. Since only a proportion of the cell population carries a mutation in somatic mosaicism, we can hypothesize that patients with somatic mosaicism might have a milder phenotype. Failure of mutation detection can also be attributed to mutations in regulatory regions of the genes. Mutations that alter gene expression, regulation, or mRNA stability rather then inactivate the protein might have less drastic phenotypic effect. Such mutations are not screened for in routine diagnostic mutation analysis. As discussed in Chapter 3.a. specific mutations in other genes involved in the mTOR signaling pathway might also result in similar phenotypes. Such mutations would dysregulate the hamartin-tuberin complex rather than inactivate the complex completely presumably resulting in milder phenotypes.

de Novo vs. Familial

We showed more severe and/or more frequent clinical manifestations associated with *de novo* cases compared to familial cases [10]. Eleven of the 17 investigated clinical features had lower frequencies in familial cases. Although in *de novo* cases, *TSC1* mutations were associated with milder phenotypes, in familial cases there was less difference between the clinical features of the groups of patients with a *TSC1* or *TSC2* mutation, suggesting that familial cases with a *TSC2* mutation are associated with a milder TSC phenotype than *de novo TSC2* cases. Phenotypes associated with *TSC1* mutations did not significantly differ in familial and *de novo* cases however. Interestingly, the proportion of *TSC1* mutations in familial cases was also higher than in *de novo* cases. In summary, mutations with milder phenotypic effects have less effect on reproductive fitness. Individuals carrying these mutations have a higher chance of reproduction and passing on the mutation, which also helps explain the higher proportion of *TSC1* mutations in familial cases.

Phenotypic Variability in TSC

Another important aspect that has to be covered under the title phenotypic expression of TSC is the broad phenotypic variability among TSC patients. Even within families the expression of the disease can be highly variable [60]. Studies conducted on monozygotic twins who carry the exact same genetic information and the exact same germline mutation suggest that TSC phenotype is not solely determined by genetic factors (the germline mutation, modifying genes, etc.) [61-64]. Although monozygotic twins carry the exact same genetic information, most are not identical due to intrinsic and extrinsic factors, including: differences in placenta and amniotic sac, vascularization of the separate cell masses, genetic modifications that occur after the separation of twins and

epigenetic modifications [65]. Epigenetic information consists mainly of DNA methylation and histone modifications, and controls heritable states of gene expression. A recent study revealed that the patterns of epigenetic modifications were different in monozygotic twins who were older, had different lifestyles, and had spent less of their lives together, owing both to environmental factors (smoking, physical activity, diet, etc.) and to a process called “epigenetic drift” which is the accumulation of small defects in transmitting epigenetic information through successive cell divisions, or maintaining it in differentiated cells.

Another factor that plays a role in phenotypic variability is probably the effects of disease modifying genes [66]. Modifier genes are genes other than the disease-causing gene(s), which influence the expression of a disease. Products of modifier genes can affect splicing, transcription, translation, protein trafficking, posttranslational processes, degradation or secretion, functioning primarily in cells where the casual or primary gene(s) is expressed. Alternatively, modifier genes may regulate responses like inflammation, fibrosis, host defense, synaptic excitability, vascularization or virtually any other response to a disturbance of homeostasis, in this case functioning in cells other than those primarily affected.

One of the best-studied diseases for the effects of modifying genes is cystic fibrosis (CF) [67]. CF is an autosomal recessive single-gene disorder caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) gene. The gene encodes a multi-domain protein, a small chloride channel that is found at the apical border of epithelial cells lining the airways, pancreatic ducts, sweat ducts, intestines, biliary tree and vas deferens. Although correlation between some types of mutation and disease severity is evident, there is substantial phenotypic variability even between patients who have identical *CFTR* mutations. Environmental factors as well as many disease modifying genes (1-acting on the basic molecular level, such as providing alternative chloride conductance, and regulating splicing and expression of the *CFTR* gene, 2-modulating susceptibility to infection and inflammatory response, 3-regulating mucociliary clearance and epithelial tissue damage repair, or 4-modulating proteolysis and fibrosis) have been shown to be associated with the phenotype [66]. In a recent study, more than 800 patients who were homozygous for the ΔF508 mutation were genotyped for 16 polymorphisms in 10 genes that were reported by others as modifiers of disease severity in cystic fibrosis [68]. Significant allelic and genotypic associations with phenotype were only seen for *TGFβ1*. This study illustrates that a very large number of patients with the same genotype is necessary to demonstrate significant associations between disease modifiers and phenotypes.

Neurofibromatosis type 1 (NF1), an autosomal dominantly inherited disorder that closely resembles TSC and is caused by mutations in the tumor suppressor gene *NF1*, has also variable phenotypic expression and lacks clear genotype-phenotype correlations [69]. The protein product of the *NF1*, neurofibromin functions as a GAP towards the small GTPase Ras. LOH and mutation analysis for somatic “second hit” mutations revealed that while in some patients there are predominantly large rearrangements leading to LOH, in others there are predominantly point mutations and small deletions and insertions [70, 71]. The finding that the same type of second hit mechanism is frequently involved in the same patient suggests that these mechanisms are controlled by genetic

factors influencing somatic recombination and mismatch repair [70]. Genes regulating these pathways might be disease modifying for NF1. A patient with a germline *NF1* mutation in addition to decreased mismatch repair capability or increased mutation rate during mitotic recombination due to less thorough recombination machinery might have a more severe phenotype compared to another patient with the same mutation but a higher mismatch repair capability or more efficient mitotic recombination. Similar to NF1, the efficiency of mismatch repair as well as somatic recombination rate and accuracy undoubtedly play a role in the frequency of somatic "second hit" events and hence in phenotypic expression of TSC.

One of the most striking observations from our cohort was maybe the phenotypic difference between males and females [10]. Fourteen of 17 clinical manifestations of TSC were more frequent in males. Males were more likely to be mentally retarded. In addition, facial angiofibromas, retinal phakomas, retinal depigmentations and gingival fibromas were significantly more frequent among males. Smalley et al. have shown that males with TSC are more likely to be autistic or have learning disability compared to females with TSC [72]. In addition, estrogen receptor α (ERα), ERβ and androgen receptor have been shown to be expressed on cells derived from LAM-associated AML [73]. Sex hormones have been shown to stimulate the growth of these cells and activate both genomic and nongenomic signaling pathways. A mouse model of *TSC1* revealed more common, more severe liver hemangiomas and higher mortality in females [74]. Expression of both ERα and PR was found in the liver hemangiomas of this *TSC1* mouse model [75]. In addition, estrogen treatment increased frequency and severity of liver hemangiomas in both female and male mice. On the other hand, tamoxifen significantly reduced the incidence and severity of liver hemangiomas in female mice. In line with these data, lymphangioleiomyomatosis affects exclusively females. This condition is either a rare sporadic disease or a clinical manifestation of TSC [76]. Primary mutations in sporadic LAM are almost exclusively somatic *TSC1* or *TSC2* mutations (except one apparently sporadic LAM case with a germline *TSC1* mutation [77]), while TSC associated LAM is the result of germline *TSC* mutations. The occasional improvement of clinical symptoms of LAM by oophorectomy, progesterone, or tamoxifen [78, 79], the detection of estrogen and progesterone receptors in LAM tissues [80], and the development or worsening of LAM during pregnancy or exogenous estrogen therapy [78], all suggest that female sex hormones have a role in the pathogenesis of LAM. These differences in phenotypic expression of TSC between males and females might be associated with a disease modifying effect of sex hormones or cellular pathways involving steroid hormones.

Finding disease modifying genes for TSC will not be an easy task. Even for a disease like CF where there are many patients with the same homozygous mutation, the majority of the studies were conducted on rather small numbers of cases. Most of these studies were not repeated and, if they were, often led to conflicting results [66]. One of the major problems is collecting sufficient numbers of patients with comparable genotypes and adequately classified phenotypes for a whole genome type of scan [81]. "Candidate gene" strategies, which search for associations between relevant genes and disease phenotypes, will be the preferred method for identifying disease modifying genes in TSC. However, since the candidate gene approach is one of association and not of "cause and effect", there will be associations by chance because of the huge number of comparisons required. Candidate genes will probably include the genes encoding proteins involved in the mTOR signaling

pathway. In addition, genes specifically up- or down-regulated in TSC-associated lesions, found using genomic and/or proteomic approaches [82-86], might also be involved in overall phenotypic expression of TSC.

In short, phenotypic expression of TSC is probably the product of the gene involved (*TSC1* vs. *TSC2*), the nature, type and effect of the primary mutation (germ-line mutation vs. somatic mosaic mutation, truncating mutation, missense mutation with residual activity, large rearrangement, etc.), the nature, type and effect of the second hit (LOH, point mutations, small deletions, 5' end mutation, 3' end mutation, large deletion involving other genes, etc.), modifying genes (genes involving mismatch repair or mitotic recombination efficiency and accuracy, transcription factors, genes involved in sex hormone-related pathways, etc.) and environmental factors (radiation, smoking, eating habits, alkylating agents, oxidative stress, etc.).

3.d. Genetic Counseling and Prenatal Diagnosis in TSC

TSC is an autosomal dominantly inherited disorder with highly variable clinical expression, which seriously complicates genetic counseling. Penetrance is considered to be complete, although recently mutation carriers have been identified who do not meet the formal diagnostic criteria [87] (Chapters 2.d and 2.e; further discussed under Chapter 3.c and below). Apparently, some mutations are associated with a clinical spectrum that differs from the average. These mutations may confer a (relatively mild) TSC phenotype to some and only minor signs to others. Clinical follow up of patients and presently unaffected mutation carriers will be needed to confirm this, which may lead to a different assessment of TSC penetrance. These developments will assist genetic counseling in some families. However, approximately two thirds of the cases are caused by *de novo* mutations, which are often unique and knowledge on their effects is not available. In addition, germline mosaicism with or without accompanying somatic mosaicism is well documented for TSC. The risk of a healthy couple with an affected offspring to have another child with TSC is calculated to be approximately 2% when germline mosaicism is taken into account, while in case of parents with signs and symptoms of the disease the risk of having an affected child increases substantially, up to 50%. TSC patients have an inherent 50% probability of passing on the disease to their offspring.

There are eight clinical genetic centers / academic Departments of Clinical Genetics in the Netherlands and in all these centers genetic counseling for every genetic disease is given. For TSC, individuals are referred to these centers by either their general practitioners or specialists, in case TSC is diagnosed or suspected in them or their families. Patients and their relatives are informed about the genetic aspects of TSC and are presented with the possibility and the consequences of mutation analysis. The Department of Clinical Genetics, Erasmus MC, has the only DNA diagnostic laboratory in the Netherlands that offers mutation analysis of the *TSC* genes. We receive genetic material for TSC mutation analysis from these centers as well as the specialized TSC outpatient clinic in Utrecht, specialists from the Netherlands and many other countries.

At present, a pathogenic mutation can be identified in approximately 85% of the definite TSC

patients, facilitating diagnosis of other affected relatives and tailored genetic counseling for these families [10]. In cases where the clinical information is doubtful or unavailable, mutation analysis (in combination with functional analysis) may also assist in establishing the diagnosis in an index patient (Chapters 2.d and 2.e). In addition to mutation analysis, detailed TSC clinical work-up on parents is also routinely performed to optimize risk calculations for the family.

In a genetic disorder with complete penetrance, like TSC is suggested to be, one would expect all individuals carrying a pathogenic mutation to be affected. However, we have shown in a large family that many individuals carrying a pathogenic mutation did not fulfil the clinical diagnostic criteria for TSC (Chapter 2.d) and would not have been diagnosed as TSC patients if a pathogenic mutation was not identified or TSC did not segregate in the family. In order to minimize false negative diagnosis, “having an affected family member” could have been included in the clinical diagnostic criteria. However, this would have resulted in the false diagnosis of six individuals as TSC patients in the very same family (Chapter 2.d). In another family (family 2), this would have led to false diagnosis of two individuals (II:8 and II:10) as TSC patients and would have excluded the pathogenic mutation in the family as the disease-causing mutation (Chapter 2.e). On the other hand, in this family (family 2) and in yet another family (family 1) the fathers shown to be carrying pathogenic mutations were unaffected by TSC (Chapter 2.e).

It must be noted that a mutation in either TSC gene is a genetic defect that might lead to signs and symptoms of the disease and that the disease TSC is a clinical entity characterized by the occurrence of certain signs and symptoms. Hence, it is possible to identify a pathogenic mutation in an apparently unaffected individual during mutation analysis of a family with a symptomatic proband. Such an individual, although unaffected at the time of identification of the mutation, might have an increased risk of developing various TSC-associated lesions and have an up to 50% risk of having affected offspring. However, when the current diagnostic criteria are used, such individuals are considered to be unaffected by TSC. Either such individuals are presymptomatic TSC patients or the penetrance of TSC is not complete. Another possibility is that the currently used clinical criteria are not sufficient to diagnose all affected cases. No matter how these individuals are classified clinically, they have to be monitored as if they were TSC patients. Identification of a mutation in an apparently unaffected individual can have social and legal consequences as well. Fortunately, in the Netherlands, the rights of the individual are protected by law and the results of a genetic test cannot be requested by employers or insurance companies. If the results however are made available, it must be made clear that the individual was not affected by the disease at the time of the genetic test.

On the other hand, approximately 15% of the TSC patients seem to have no mutation in the *TSC1* or *TSC2* genes [6, 7, 10]. As previously discussed (Chapter 3.1, ‘Missed Mutations?’), it might be difficult or even impossible to detect all the mutations. Another possibility is, however, that a proportion of these cases are misdiagnosed as TSC patients. None of the TSC-associated lesions are pathognomonic for TSC and these can have a diverse etiology. Although many are not frequently seen in the general population, combinations of lesions that are accepted as diagnostic of TSC will appear in some individuals by chance. In addition, as discussed previously (Chapter

3.a, 'Or a Third Locus?'), specific mutations in genes encoding different components of the mTOR signaling pathway may result in similar phenotypes. Although the molecular defect in cases where no mutation could be identified is elusive, these individuals show signs and symptoms of the clinical entity and require clinical management accordingly.

One of the goals of genetic counseling is prognostic evaluation. Studies on genotype-phenotype correlations have shown that mutations in the *TSC2* gene compared to mutations in the *TSC1* gene, *de novo* mutations compared to familial mutations, male gender compared to female gender are associated with more severe phenotypes for example [6, 7, 10]. In addition, there are already a growing number of mutations that are associated with milder phenotypes [58, 59] (Chapters 2.d and 2.e). These data might in the future be used to calculate individuals' risks for developing particular manifestations of the disease. However, the phenotypic variability within families, even between monozygotic twins, suggests that we need a better understanding of the factors determining the severity of the disease.

Cardiac rhabdomyomas, intrauterine seizures and some cerebral lesions can be detected in utero using ultrasound examination (USG). However, it must be noted that a negative USG result does not exclude the diagnosis of TSC, which limits its applicability in pregnancies known to be at risk for TSC. When these ultrasound abnormalities are observed during routine prenatal screening, in the absence of a family history of TSC, they may not be sufficient for a diagnosis of TSC in the fetus. Prenatal mutation analysis has been advocated in such cases [88]. However, the absence of an index patient with a certain diagnosis of TSC, the chance of finding a sequence variant of unknown significance and the restricted availability of time complicate this approach.

In families where a mutation has been identified in the index patient or where linkage analysis can be performed, prenatal DNA diagnosis can be offered for future pregnancies. In our center we received prenatal testing requests from 16 TSC families for a total of 23 pregnancies. In one of these families the prenatal tests were done before the identification of the pathogenic mutation and linkage analysis was applied. In five pregnancies the fetus was found to be carrying the disease-causing mutation/allele. In two of these cases the pregnancy was terminated, however the follow-up data for the other three cases was not available. Except a few centers in Europe including our department, prenatal DNA testing for TSC is not routinely offered. For disorders with wide phenotypic variability such as TSC, the decision to terminate a pregnancy could be very difficult for the parents, especially if the parent who carries the mutation is only mildly affected. On the other hand, having a severely affected child might influence the parents against another pregnancy.

Although termination of a pregnancy is socioeconomically and medically justified when a fetus affected with TSC is identified by prenatal testing, the burden of therapeutic abortion on the family is immense and even unacceptable for some religious/ethnic groups. In addition, the risk of having an affected fetus for each pregnancy is independent of each other. Because of these reasons, preimplantation genetic diagnosis (PGD) might be an alternative for TSC families. In PGD, genetic testing is performed on single blastomeres obtained from 3-day-old embryos after in vitro fertilization (IVF) and only embryos diagnosed as disease-free are transferred to the mother in order

to avoid therapeutic abortion. There are disadvantages associated with PGD as well, such as the requirement for IVF and subsequent embryo biopsy. In addition, an efficient and reliable single-cell PCR-based assay for PGD is labor-intensive and expensive. For disorders with common mutations, mutation specific assays can be developed which is impractical for TSC. Multiplex PCR based protocols for closely linked genetic markers seem to be more appropriate for the *TSC1* and *TSC2* loci. This approach would require the previous identification of the disease causing (*TSC1* or *TSC2* gene) mutation in most families, because TSC families are rarely large enough to allow a reliable locus assignment by linkage analysis. Some of the technical difficulties related to PCR on single cells are allele-specific amplification failure or allele drop-out (ADO), recombination events and contamination, which may all theoretically lead to misdiagnosis. The misdiagnosis rate related to these problems can be minimized by the simultaneous amplification of highly polymorphic markers linked to the genes and/or analyzing two blastomeres instead of one. In cases where a mutation-specific protocol is necessitated, to prevent misdiagnosis in addition to exclusion of the mutant allele, the presence of the normal alleles has to be shown using linked polymorphic markers as well [89]. For maternally derived mutations, testing of the first and second polar bodies will also increase the reliability of PGD. Recently, Spits et al published their PGD protocols for six families with neurofibromatosis type 1 [90]. The authors used multiplex PCR for informative markers in four families and a mutation-specific protocol in combination with an informative marker in the other two families.

3.e. Functions of the *TSC1* and *TSC2* Gene Products

Tuberous sclerosis complex (TSC) is an autosomal dominant, multi-system disorder characterized by *hamartias*, *hamartomas* and *hamartoblastomas*. The hallmarks of these lesions are the dysplastic, undifferentiated cells.

The recognition of these cells have led to the hypothesis that the *TSC1* and *TSC2* gene products, hamartin and tuberin respectively, have important cellular functions in division, size control, differentiation and/or migration.

The similar phenotypes associated with mutations in the *TSC1* or *TSC2* genes have led to the hypothesis that hamartin and tuberin might form a complex. Subsequently, hamartin and tuberin were shown to interact with each other *in vivo*. Both genes act as tumor suppressor genes as shown by LOH in many TSC-associated lesions. Until recently, the cellular functions of hamartin and tuberin and the cellular pathway(s) they are involved in remained a mystery.

In Drosophila, *TSC1* was uncovered in several genetic screens aimed at identifying regulators of cell size [91, 92]. Other studies confirmed that hamartin-tuberin complex acts upstream of mTOR and downstream of PI3K and Akt/PKB [35, 36, 93-96]. Shortly thereafter, the direct target of the complex, rheb, was also placed in the growing mTOR pathway [27, 28, 97], a more detailed overview of which is presented in figure 2. Dysregulation of the mTOR signaling pathway has been shown in various cell types in TSC associated lesions, in TSC animal models, and in *TSC1* -/- or *TSC2* -/-

cells. In addition to PI3K and Akt/PKB pathway, protein kinase C (PKC)/MAPK signaling pathway has also been shown to relay its input through the hamartin-tuberin complex [98, 99].

Besides S6K and 4EBP-1, the well-known effectors of the mTOR signaling pathway, it has recently been shown that mTOR-mediated signaling also enhances the expression of HIF-1α [100-102], a transcription factor that is normally expressed and becomes more stable under hypoxic conditions [103], which in turn results in the concomitant increase in the production and secretion of vascular endothelial growth factor (VEGF), a cytokine that induces angiogenesis. The angiogenic response induced by VEGF is a common effect observed in many tumors, also tumors not associated with TSC, and is necessary for tumor growth [104].

The hamartin-tuberin complex also seems to couple translation and cell size regulation to cell cycle progression (See Chapter 1, table 5). In addition, tuberin has been shown to interact with all isoforms of 14-3-3 proteins [105-111], which are abundant, widely expressed acidic polypeptides that bind to phosphoserine-/ phosphothreonine-containing motifs in a sequence-specific manner and function as adaptor molecules modulating interactions/functions of components involved in signal transduction and in cell cycle control [112].

It was recently noted that rapamycin, a potent inhibitor of mTOR (see Chapter 3.f., 'Clinical Management'), had limited effect on VEGF secretion in *Tsc2* -/- cells [113], on cyclin D1 and β-catenin expression in the Eker rat renal tumors [114] and on reducing the number of precursor lesions during the initiation phase of renal tumorigenesis in the Eker rat [115] suggesting the involvement of β-catenin signaling pathway in TSC-associated pathology. This pathway has been implicated in multiple cellular processes such as cell proliferation, survival, differentiation, polarity, and migration, all of which are relevant to TSC pathology. Biochemical analyses revealed that the hamartin-tuberin complex regulates β-catenin stability and protein expression, inhibits Wnt-induced β-catenin-dependent transcriptional activity, and interacts with the GSK3-degradation complex [116].

Another interesting finding was the interaction between tuberin and the E6 oncoprotein of the human papillomavirus (HPV16 E6) which leads to the proteasome mediated degradation of tuberin and to activation of mTOR signaling pathway [117]. Human papillomaviruses infect epithelial cells, induce epithelial tumors and benign lesions, and the E6 protein is required for this process [118, 119]. Tuberin and/or hamartin could be the target of other (viral) oncoproteins as well, since adjustments in cellular processes and extensive resources are necessary for tumor growth and maintenance, including protein synthesis, (neo-)angiogenesis, uncoupling of cell growth and cell cycle. The hamartin-tuberin complex is involved in many of these processes and deactivation of the complex could be a prerequisite for oncogenic transformation.

Hamartin has also been shown to interact specifically with neurofilament light chain (NF-L) [120]. NF-L is an intermediate filament, initially expressed early in neuronal differentiation [121]. The co-localization of hamartin and tuberin with NF-L is particularly enriched in the proximal and central regions of neuronal growth cones [122]. An earlier study showed that the interaction of hamartin with the ERM (ezrin/radixin/moesin) family of actin-binding proteins was necessary for Rho activation

[120, 123]. The ERM family of proteins has been shown to regulate important aspects of growth-cone development and maintenance, modulating neurite formation and polarity [124, 125]. The interaction of hamartin with NF-L suggests that hamartin could function as a novel integrator of the neuronal cytoskeleton [122]. It is possible that through its interactions with NF-L and ERM proteins, hamartin plays an important role in neuronal migration, which could explain certain neuronal differentiation and migration defects observed in TSC patients.

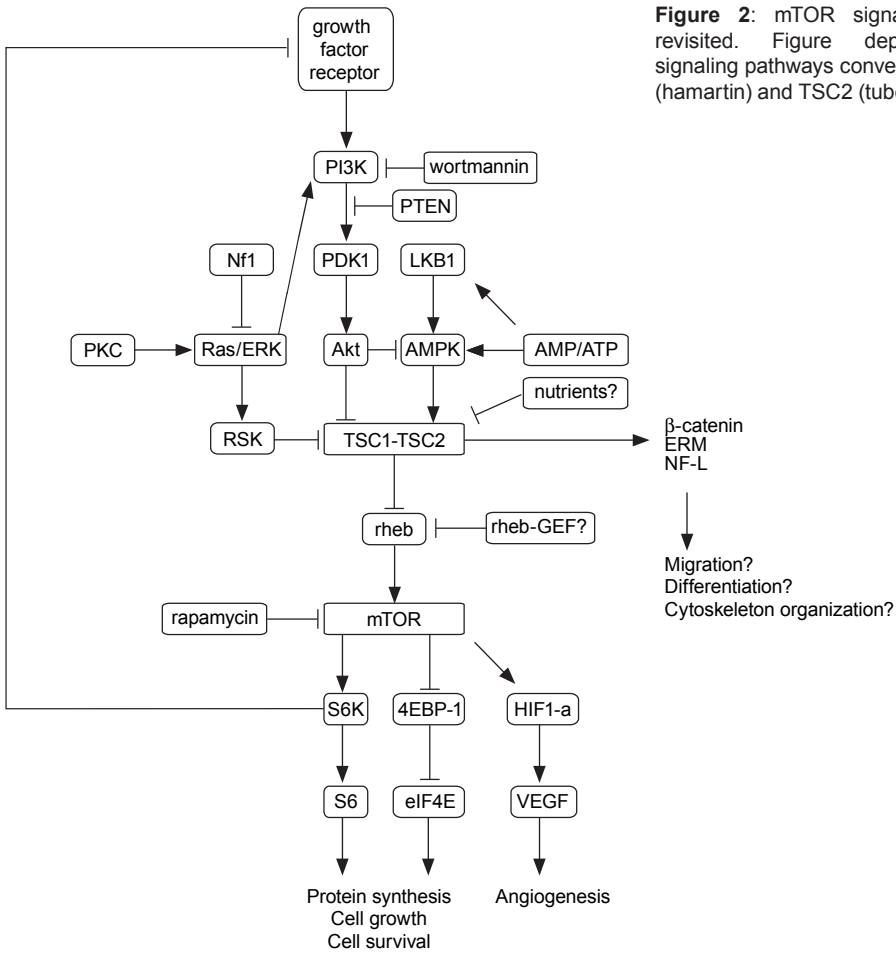


Figure 2: mTOR signaling pathway revisited. Figure depicts various signaling pathways converging on TSC1 (hamartin) and TSC2 (tuberin).

In summary, the hamartin-tuberin complex through its regulatory role in different signaling pathways seems to be of significant importance for the orchestrating of various cellular processes. In addition to their role in TSC-associated pathology, *TSC* genes can also be involved in various tumors that are not associated with TSC and in various developmental lesions. A better understanding of the functions of hamartin and tuberin and the cellular pathways they are involved in might yield many positive results including better treatment of various tumor types, insight into neuronal development, differentiation and migration, and the pathologies associated with these processes.

3.f. Concluding Remarks and Future Directions

Mutation Analysis

To minimize human-error and increase throughput, automated systems are becoming more common in diagnostic facilities around the world. In our laboratory as well, instead of the labor-intensive prescreening tests like DGGE and SSCP, we now use semi-automated direct sequencing to search for small nucleotide changes. For larger rearrangements, we have started using MLPA as a screening test, which is also a semi-automated PCR-based system. However, a copy number change identified by this assay needs to be confirmed by another, independent method, such as long-range PCR, FISH or Southern blotting.

For cases where we fail to identify a pathogenic mutation, since somatic mosaicism might be responsible for at least a proportion of these cases, mutation analysis can be applied to DNA isolated directly from TSC-associated lesions. Detecting the expression of hamartin and tuberin and/or the levels of *TSC1* and *TSC2* transcripts could also give an impression as to where the pathogenic mutation is located. In addition, in larger families with multiple affected members, linkage analysis is recommended to point the search for the pathogenic mutation in the right direction.

In addition to routine mutation analysis, prenatal DNA testing should be accessible for all TSC families. Preimplantation genetic diagnosis (PGD) strategies should be developed for TSC and patients should be informed about the possibility of PGD.

The Hamartin-Tuberin Complex

The hamartin-tuberin complex acts as a GTPase activating protein (GAP) towards rheb to regulate mTOR signaling pathway and this pathway plays a major role in TSC-associated pathology. However, the guanine-nucleotide exchange factor (GEF) for rheb has not yet been identified. Recently, Nellist et al. using mass spectrometry, showed that DOCK7 interacts with hamartin [126]. DOCK7 is a 240kDA member of CDM protein family of GEFs, the molecular target of which is not yet known. Further research will clarify whether DOCK7 acts as a GEF for rheb, and if proven, DOCK7 might be another molecular target for the therapy of TSC-associated lesions.

Another important functional aspect that needs to be addressed is the molecular pathogenesis of cortical tubers. It is still uncertain whether a second hit event is necessary for the development of cortical tubers. Genetic material isolated from a tuber, however, also contains genetic material from the surrounding normal tissue. Isolation of single giant cells with microdissection microscopy will help determine whether second hits play a role in the pathogenesis. It is also possible that haploinsufficiency is the cause of such lesions. Another possibility is the inactivation of the hamartin-tuberin complex through another mechanism (e.g. phosphorylation) [56, 57]. In addition, the possible signaling pathways that lead to the formation of cortical tubers when dysregulated are also not yet known. To investigate the roles of hamartin and tuberin in vertebrate embryonal development, a new TSC animal model is being developed in our department. The zebrafish, *Danio* (*D.*) *rerio*, is a tropical freshwater fish with external, transparent embryos that allow visual screening at both micro- and macroscopic levels. The short generation time of the fish and the highly conserved cellular processes make *D. rerio* an excellent model organism to study early vertebrate development.

Clinical Management

As discussed earlier (Chapter 3.d. Genetic counseling and prenatal diagnosis in TSC), TSC is a clinical entity characterized by the occurrence of certain signs and symptoms. The TSC diagnosis is made, not to stigmatize individuals, but to identify those who need medical attention. In this respect, not all individuals carrying a pathogenic mutation in either *TSC1* or *TSC2* gene we have identified are TSC patients (Chapters 2.d and 2.e). A change in the diagnostic criteria is not proposed, since this will only lead to further confusion and falsely diagnosed TSC cases. Importantly, the current criteria seem adequate to identify TSC patients in need of medical attention. On the other hand, an apparently unaffected individual carrying a pathogenic mutation, no matter how such an individual is classified clinically (presymptomatic TSC patient, asymptomatic TSC carrier, etc.) needs to be monitored regularly for signs and symptoms of the disease. Such individuals might have an increased risk of developing various TSC-associated lesions and an up to 50% risk of having affected offspring. On the other side of the spectrum are the TSC patients where we are unable to identify disease-causing mutations. Some of these cases probably have somatic mosaicism, the rate of which is below our current detection limits. However, others might have mutations in other genes related directly or indirectly to TSC. Although the exact molecular defect in such cases is not known, such patients are TSC patients and should be treated accordingly.

The next step in the clinical research will be to determine the factors that affect the clinical outcome besides the primary mutation. What is the biological basis of the phenotypic heterogeneity in TSC? Answering this question will require even larger cohorts, patients with adequately defined phenotypes and similar genotypes, investigations of the nature, type and effect of the second hit, and study of the effects of possible modifier genes.

After the role of the hamartin-tuberin complex was identified in the mammalian target of rapamycin (mTOR) signaling pathway, rapamycin immediately became the focus of attention for researchers and clinicians working on TSC.

Rapamycin is an antifungal agent, a macrocyclic lactone, produced by the *Streptomyces hygroscopicus* [127]. This drug has been used in transplantation medicine as an immunosuppressive agent to treat acute rejection episodes for over a decade now. Rapamycin binds to the cytosolic immunophilin FKBP12, forming a complex that specifically inhibits mTOR [128]. The inhibition of mTOR results in inhibition of proliferative and differentiation signals delivered by cytokines including interleukins (IL)-2, -3, -5, -6, and -15, as well as the hematologic and vascular families of humoral growth factors. In addition, rapamycin interrupts B cell responses, reducing immunoglobulin production [129-131].

Besides its use in transplantation medicine, rapamycin is also an attractive anti-cancer agent since the mTOR signaling pathway is constitutively activated in many types of human cancer [132, 133]. However, many cancer cells are resistant to rapamycin and its derivatives. Recently it was shown that rapamycin treatment results in the phosphorylation and activation of Akt and eIF4E, which may contribute to this resistance [134]. The activation of S6K leads to a feedback inhibition of PI3K/

Akt through phosphorylation and degradation of IRS-1, while inhibition of S6K phosphorylation by rapamycin breaks this negative feedback loop [135, 136]. Using a PI3K inhibitor (LY294002) in combination with rapamycin greatly enhances the inhibitory effects on the growth and colony formation of cancer cells [134].

Rapamycin is also suggested as a therapeutic agent for the tumor predisposition syndrome, neurofibromatosis type 1 (NF-1) [137]. Although activation of PI3K/Akt has previously been shown in NF-1 associated tumors, the exact pathogenic targets of this pathway were unknown until recently [138, 139]. Shortly after the demonstration of hyperactivation of the mTOR signaling pathway using a proteomic approach [140], NF1 deficient malignant human tumor cell lines were shown to be highly sensitive to rapamycin treatment [137]. Neurofibromin, the protein product of the *NF1* gene, is a Ras GTPase activating protein (GAP). The activation of the mTOR signaling pathway in NF1 deficient cells is suggested to be the result of increased Ras activation, which in turn activates PI3K and Akt. The mTOR signaling pathway is also an attractive therapeutic target for other hamartomatous diseases including Cowden disease and Peutz-Jeghers syndrome.

After studies in animal models, clinical trials with rapamycin have been initiated in TSC and LAM patients [114, 115, 141-143]. In the Eker rat, a very well-studied *Tsc2* animal model, administration of rapamycin and its analogs resulted in induction of apoptosis and reduction of cell proliferation in renal tumors [114]. Rapamycin has also improved the clinical state and survival associated with pituitary tumors seen in Eker rats [115]. In addition, it has been shown effective in reducing the severity and number of renal cystadenomas in *Tsc2*+/- mice [144]. Embryonic fibroblast cell lines prepared from *Tsc1* and *Tsc2* null mice also show a dramatic normalization of mTOR signaling after treatment with rapamycin [145].

During the TSC International Research Conference (2004, Cambridge, UK) preliminary information about clinical trials on TSC and LAM patients was presented. One patient with two renal AMLs, one of constant size and one that increased in volume over four years, was treated with rapamycin and the active AML has been shown to be 71% reduced in size within six months [141]. However, no changes were observed in the AML of constant size. At the same conference, preliminary data of larger clinical trials with rapamycin were also presented showing promising results, which include improvement in the vital lung capacity of patients with LAM and reduction of AML tumor volume in both LAM and TSC patients [142, 143].

However, rapamycin does not cross the blood-brain barrier, and the benefit of rapamycin or rapamycin analogs in the treatment of developmental, cognitive and psychiatric manifestations of TSC needs yet to be investigated. Early intervention and symptomatic treatment (e.g. seizure control) might be more important and help decrease the burden of the disease for such manifestations. In addition, prolonged rapamycin administration might result in drug resistance [115]. Although only seen in a minority of lesions, the mechanism of drug resistance and ways to circumvent this problem require immediate scientific and clinical attention. Using rapamycin in combination with other chemical agents could help circumvent this particular problem.

IFN-γ-Jak-STAT signaling has also been shown to be perturbed in TSC-associated lesions and cell lines [145], and recently it was shown in *Tsc2*+/- mice that IFN-γ treatment can reduce the severity of kidney cystadenomas and liver hemangiomas [144]. Importantly, rapamycin and IFN-γ have different mechanisms of action and the combination of rapamycin and IFN-γ may have additive beneficial effects.

In addition to the mTOR signaling pathway, there are as yet therapeutically unexplored signaling pathways in which hamartin-tuberin complex plays a role. However, the relevance of these pathways to TSC-associated pathology remains to be elucidated.

Further studies of the pathways that include the hamartin-tuberin complex and its regulation will yield better therapeutic options for a growing number of disorders.

References

1. *Identification and characterization of the tuberous sclerosis gene on chromosome 16. The European Chromosome 16 Tuberous Sclerosis Consortium.* Cell, 1993. **75**(7): p. 1305-15.
2. van Slegtenhorst, M., et al., *Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34.* Science, 1997. **277**(5327): p. 805-8.
3. van Bakel, I., et al., *Mutations in the TSC2 gene: analysis of the complete coding sequence using the protein truncation test (PTT).* Hum Mol Genet, 1997. **6**(9): p. 1409-14.
4. van Slegtenhorst, M., et al., *Mutational spectrum of the TSC1 gene in a cohort of 225 tuberous sclerosis complex patients: no evidence for genotype-phenotype correlation.* J Med Genet, 1999. **36**(4): p. 285-9.
5. Au, K.S., et al., *Germ-line mutational analysis of the TSC2 gene in 90 tuberous-sclerosis patients.* Am J Hum Genet, 1998. **62**(2): p. 286-94.
6. Jones, A.C., et al., *Comprehensive mutation analysis of TSC1 and TSC2-and phenotypic correlations in 150 families with tuberous sclerosis.* Am J Hum Genet, 1999. **64**(5): p. 1305-15.
7. Dabora, S.L., et al., *Mutational analysis in a cohort of 224 tuberous sclerosis patients indicates increased severity of TSC2, compared with TSC1, disease in multiple organs.* Am J Hum Genet, 2001. **68**(1): p. 64-80.
8. Niida, Y., et al., *Analysis of both TSC1 and TSC2 for germline mutations in 126 unrelated patients with tuberous sclerosis.* Hum Mutat, 1999. **14**(5): p. 412-22.
9. Langkau, N., et al., *TSC1 and TSC2 mutations in tuberous sclerosis, the associated phenotypes and a model to explain observed TSC1/ TSC2 frequency ratios.* Eur J Pediatr, 2002. **161**(7): p. 393-402.
10. Sancak, O., et al., *Mutational analysis of the TSC1 and TSC2 genes in a diagnostic setting: genotype--phenotype correlations and comparison of diagnostic DNA techniques in Tuberous Sclerosis Complex.* Eur J Hum Genet, 2005. **13**(6): p. 731-41.

11. Mayer, K., W. Ballhausen, and H.D. Rott, *Mutation screening of the entire coding regions of the TSC1 and the TSC2 gene with the protein truncation test (PTT) identifies frequent splicing defects.* Hum Mutat, 1999. **14**(5): p. 401-11.
12. Kwiatkowski, D., *TSC1, TSC2, TSC3? Or mosaicism?* Eur J Hum Genet, 2005. **13**(6): p. 695-6.
13. Longa, L., et al., *TSC1 and TSC2 deletions differ in size, preference for recombinatorial sequences, and location within the gene.* Hum Genet, 2001. **108**(2): p. 156-66.
14. Sellner, L.N. and G.R. Taylor, *MLPA and MAPH: new techniques for detection of gene deletions.* Hum Mutat, 2004. **23**(5): p. 413-9.
15. Mayer, K., et al., *Three novel types of splicing aberrations in the tuberous sclerosis TSC2 gene caused by mutations apart from splice consensus sequences.* Biochim Biophys Acta, 2000. **1502**(3): p. 495-507.
16. Kluwe, L. and V.F. Mautner, *Mosaicism in sporadic neurofibromatosis 2 patients.* Hum Mol Genet, 1998. **7**(13): p. 2051-5.
17. Erickson, R.P., *Somatic gene mutation and human disease other than cancer.* Mutat Res, 2003. **543**(2): p. 125-36.
18. Sampson, J.R., et al., *Renal cystic disease in tuberous sclerosis: role of the polycystic kidney disease 1 gene.* Am J Hum Genet, 1997. **61**(4): p. 843-51.
19. Roberts, P.S., et al., *Somatic mosaicism is rare in unaffected parents of patients with sporadic tuberous sclerosis.* J Med Genet, 2004. **41**(5): p. e69.
20. Verhoef, S., et al., *Somatic mosaicism and clinical variation in tuberous sclerosis complex.* Lancet, 1995. **345**(8943): p. 202.
21. Verhoef, S., et al., *High rate of mosaicism in tuberous sclerosis complex.* Am J Hum Genet, 1999. **64**(6): p. 1632-7.
22. Jones, A.C., J.R. Sampson, and J.P. Cheadle, *Low level mosaicism detectable by DHPLC but not by direct sequencing.* Hum Mutat, 2001. **17**(3): p. 233-4.
23. Kwiatkowska, J., et al., *Mosaicism in tuberous sclerosis as a potential cause of the failure of molecular diagnosis.* N Engl J Med, 1999. **340**(9): p. 703-7.
24. Sampson, J.R., et al., *Evidence for genetic heterogeneity in tuberous sclerosis.* J Med Genet, 1989. **26**(8): p. 511-6.
25. Smith, M., et al., *Mapping of a gene determining tuberous sclerosis to human chromosome 11q14-11q23.* Genomics, 1990. **6**(1): p. 105-14.
26. Northrup, H., *Tuberous sclerosis complex: genetic aspects.* J Dermatol, 1992. **19**(11): p. 914-9.
27. Garami, A., et al., *Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2.* Mol Cell, 2003. **11**(6): p. 1457-66.
28. Tee, A.R., et al., *Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb.* Curr Biol, 2003. **13**(15): p. 1259-68.
29. Lim, W., et al., *Further observations on LKB1/STK11 status and cancer risk in Peutz-Jeghers syndrome.* Br J Cancer, 2003. **89**(2): p. 308-13.

30. Inoki, K., M.N. Corradetti, and K.L. Guan, *Dysregulation of the TSC-mTOR pathway in human disease.* Nat Genet, 2005. **37**(1): p. 19-24.
31. Corradetti, M.N., et al., *Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome.* Genes Dev, 2004. **18**(13): p. 1533-8.
32. Shaw, R.J., et al., *The LKB1 tumor suppressor negatively regulates mTOR signaling.* Cancer Cell, 2004. **6**(1): p. 91-9.
33. Eng, C., *PTEN: one gene, many syndromes.* Hum Mutat, 2003. **22**(3): p. 183-98.
34. Manning, B.D., et al., *Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway.* Mol Cell, 2002. **10**(1): p. 151-62.
35. Inoki, K., et al., *TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling.* Nat Cell Biol, 2002. **4**(9): p. 648-57.
36. Potter, C.J., L.G. Pedraza, and T. Xu, *Akt regulates growth by directly phosphorylating Tsc2.* Nat Cell Biol, 2002. **4**(9): p. 658-65.
37. Zhou, X.P., et al., *Germline inactivation of PTEN and dysregulation of the phosphoinositol-3-kinase/Akt pathway cause human Lhermitte-Duclos disease in adults.* Am J Hum Genet, 2003. **73**(5): p. 1191-8.
38. Marignani, P.A., *LKB1, the multitasking tumour suppressor kinase.* J Clin Pathol, 2005. **58**(1): p. 15-9.
39. Brugarolas, J., et al., *Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex.* Genes Dev, 2004. **18**(23): p. 2893-904.
40. van Slegtenhorst, M., et al., *Interaction between hamartin and tuberin, the TSC1 and TSC2 gene products.* Hum Mol Genet, 1998. **7**(6): p. 1053-7.
41. Nellist, M., et al., *Characterization of the cytosolic tuberin-hamartin complex. Tuberin is a cytosolic chaperone for hamartin.* J Biol Chem, 1999. **274**(50): p. 35647-52.
42. Nellist, M., et al., *TSC2 missense mutations inhibit tuberin phosphorylation and prevent formation of the tuberin-hamartin complex.* Hum Mol Genet, 2001. **10**(25): p. 2889-98.
43. Nellist, M., et al., *Distinct effects of single amino-acid changes to tuberin on the function of the tuberin-hamartin complex.* Eur J Hum Genet, 2005. **13**(1): p. 59-68.
44. Rendtorff, N.D., et al., *Analysis of 65 tuberous sclerosis complex (TSC) patients by TSC2 DGGE, TSC1/TSC2 MLPA, and TSC1 long-range PCR sequencing, and report of 28 novel mutations.* Hum Mutat, 2005. **26**(4): p. 374-83.
45. Henske, E.P., et al., *Loss of heterozygosity in the tuberous sclerosis (TSC2) region of chromosome band 16p13 occurs in sporadic as well as TSC-associated renal angiomyolipomas.* Genes Chromosomes Cancer, 1995. **13**(4): p. 295-8.
46. Henske, E.P., et al., *Allelic loss is frequent in tuberous sclerosis kidney lesions but rare in brain lesions.* Am J Hum Genet, 1996. **59**(2): p. 400-6.
47. Henske, E.P., et al., *Loss of tuberin in both subependymal giant cell astrocytomas and angiomyolipomas supports a two-hit model for the pathogenesis of tuberous sclerosis tumors.* Am J Pathol, 1997. **151**(6): p. 1639-47.

48. Niida, Y., et al., *Survey of somatic mutations in tuberous sclerosis complex (TSC) hamartomas suggests different genetic mechanisms for pathogenesis of TSC lesions.* Am J Hum Genet, 2001. **69**(3): p. 493-503.
49. Sepp, T., J.R. Yates, and A.J. Green, *Loss of heterozygosity in tuberous sclerosis hamartomas.* J Med Genet, 1996. **33**(11): p. 962-4.
50. Smolarek, T.A., et al., *Evidence that lymphangiomyomatosis is caused by TSC2 mutations: chromosome 16p13 loss of heterozygosity in angiomyolipomas and lymph nodes from women with lymphangiomyomatosis.* Am J Hum Genet, 1998. **62**(4): p. 810-5.
51. Carbonara, C., et al., *9q34 loss of heterozygosity in a tuberous sclerosis astrocytoma suggests a growth suppressor-like activity also for the TSC1 gene.* Hum Mol Genet, 1994. **3**(10): p. 1829-32.
52. Green, A.J., M. Smith, and J.R. Yates, *Loss of heterozygosity on chromosome 16p13.3 in hamartomas from tuberous sclerosis patients.* Nat Genet, 1994. **6**(2): p. 193-6.
53. van Slegtenhorst, M., et al., *Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34.* Science, 1997. **277**(5327): p. 805-8.
54. Jansen, F.E., et al., *Differential localization of hamartin and tuberin and increased S6 phosphorylation in a tuber.* Neurology, 2004. **63**(7): p. 1293-5.
55. Crino, P.B. *Overview: Neuropathology of TSC*. in *TSC International Research Conference*. 2004. Cambridge, UK.
56. Jozwiak, J. and S. Jozwiak, *Giant cells: contradiction to two-hit model of tuber formation?* Cell Mol Neurobiol, 2005. **25**(5): p. 795-805.
57. Han, S., et al., *Phosphorylation of tuberin as a novel mechanism for somatic inactivation of the tuberous sclerosis complex proteins in brain lesions.* Cancer Res, 2004. **64**(3): p. 812-6.
58. Khare, L., et al., *A novel missense mutation in the GTPase activating protein homology region of TSC2 in two large families with tuberous sclerosis complex.* J Med Genet, 2001. **38**(5): p. 347-9.
59. O'Connor, S.E., et al., *A family with seizures and minor features of tuberous sclerosis and a novel TSC2 mutation.* Neurology, 2003. **61**(3): p. 409-12.
60. Smalley, S.L., F. Burger, and M. Smith, *Phenotypic variation of tuberous sclerosis in a single extended kindred.* J Med Genet, 1994. **31**(10): p. 761-5.
61. Gomez, M.R., N.L. Kuntz, and B.F. Westmoreland, *Tuberous sclerosis, early onset of seizures, and mental subnormality: study of discordant homozygous twins.* Neurology, 1982. **32**(6): p. 604-11.
62. Kondo, S., et al., *Discordant expression of tuberous sclerosis in monozygotic twins.* J Dermatol, 1991. **18**(3): p. 178-80.
63. Martin, N., et al., *Discordant clinical manifestations in monozygotic twins with the identical mutation in the TSC2 gene.* Clin Genet, 2003. **63**(5): p. 427-30.
64. Humphrey, A., et al., *Monozygotic twins with tuberous sclerosis discordant for the severity of developmental deficits.* Neurology, 2004. **62**(5): p. 795-8.
65. Fraga, M.F., et al., *Epigenetic differences arise during the lifetime of monozygotic twins.* Proc Natl Acad Sci U S A, 2005. **102**(30): p. 10604-9.

66. Slieker, M.G., et al., *Disease modifying genes in cystic fibrosis.* J Cyst Fibros, 2005. **4 Suppl 2**: p. 7-13.
67. Turcios, N.L., *Cystic fibrosis: an overview.* J Clin Gastroenterol, 2005. **39**(4): p. 307-17.
68. Drumm, M.L., et al., *Genetic modifiers of lung disease in cystic fibrosis.* N Engl J Med, 2005. **353**(14): p. 1443-53.
69. Arun, D. and D.H. Gutmann, *Recent advances in neurofibromatosis type 1.* Curr Opin Neurol, 2004. **17**(2): p. 101-5.
70. Serra, E., et al., *Somatic NF1 mutational spectrum in benign neurofibromas: mRNA splice defects are common among point mutations.* Hum Genet, 2001. **108**(5): p. 416-29.
71. Williams, S.V., et al., *Molecular genetic analysis of chromosome 9 candidate tumor-suppressor loci in bladder cancer cell lines.* Genes Chromosomes Cancer, 2002. **34**(1): p. 86-96.
72. Smalley, S.L., et al., *Autism and tuberous sclerosis.* J Autism Dev Disord, 1992. **22**(3): p. 339-55.
73. Yu, J., et al., *Estradiol and Tamoxifen Stimulate Lymphangiomyomatosis-Associated Angiomyolipoma Cell Growth and Activate Both Genomic and Non-Genomic Signaling Pathways.* Am J Physiol Lung Cell Mol Physiol, 2003.
74. Kwiatkowski, D.J., et al., *A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells.* Hum Mol Genet, 2002. **11**(5): p. 525-34.
75. El-Hashemite, N., V. Walker, and D.J. Kwiatkowski, *Estrogen enhances whereas tamoxifen retards development of Tsc mouse liver hemangioma: a tumor related to renal angiomyolipoma and pulmonary lymphangioleiomyomatosis.* Cancer Res, 2005. **65**(6): p. 2474-81.
76. Hancock, E., et al., *Lymphangioleiomyomatosis and tuberous sclerosis.* Respir Med, 2002. **96**(1): p. 7-13.
77. Sato, T., et al., *A patient with TSC1 germline mutation whose clinical phenotype was limited to lymphangioleiomyomatosis.* J Intern Med, 2004. **256**(2): p. 166-73.
78. Sullivan, E.J., *Lymphangioleiomyomatosis: a review.* Chest, 1998. **114**(6): p. 1689-703.
79. Castro, M., et al., *Pulmonary tuberous sclerosis.* Chest, 1995. **107**(1): p. 189-95.
80. Matsui, K., et al., *Hyperplasia of type II pneumocytes in pulmonary lymphangioleiomyoma tosis.* Arch Pathol Lab Med, 2000. **124**(11): p. 1642-8.
81. Drumm, M.L., *Modifier genes and variation in cystic fibrosis.* Respir Res, 2001. **2**(3): p. 125-8.
82. Stoyanova, R., et al., *Altered gene expression in phenotypically normal renal cells from carriers of tumor suppressor gene mutations.* Cancer Biol Ther, 2004. **3**(12): p. 1313-21.
83. Hengstschlager, M., et al., *The cellular response to ectopic overexpression of the tuberous sclerosis genes, TSC1 and TSC2: a proteomic approach.* Int J Oncol, 2005. **27**(3): p. 831-8.
84. Hengstschlager, M., et al., *Protein levels of alpha1-tubulin, protein disulfide isomerase, tropomyosins and vimentin are regulated by the tuberous sclerosis gene products.* Cancer Lett, 2004. **210**(2): p. 219-26.
85. Ess, K.C., et al., *Expression profiling in tuberous sclerosis complex (TSC) knockout mouse astrocytes to characterize human TSC brain pathology.* Glia, 2004. **46**(1): p. 28-40.

86. Rosner, M., et al., *Cell size regulation by the human TSC tumor suppressor proteins depends on PI3K and FKBP38.* Oncogene, 2003. **22**(31): p. 4786-98.
87. Kwiatkowska, J., et al., *Comprehensive mutational analysis of the TSC1 gene: observations on frequency of mutation, associated features, and nonpenetrance.* Ann Hum Genet, 1998. **62 (Pt 4)**: p. 277-85.
88. Milunsky, A., et al., *Precise prenatal diagnosis of tuberous sclerosis by sequencing the TSC2 gene.* Prenat Diagn, 2005. **25**(7): p. 582-5.
89. Kuliev, A. and Y. Verlinsky, *Preimplantation genetic diagnosis in assisted reproduction.* Expert Rev Mol Diagn, 2005. **5**(4): p. 499-505.
90. Spits, C., et al., *Preimplantation genetic diagnosis for neurofibromatosis type 1.* Mol Hum Reprod, 2005. **11**(5): p. 381-7.
91. Ito, N. and G.M. Rubin, *gigas, a Drosophila homolog of tuberous sclerosis gene product-2, regulates the cell cycle.* Cell, 1999. **96**(4): p. 529-39.
92. Tapon, N., et al., *The Drosophila tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation.* Cell, 2001. **105**(3): p. 345-55.
93. Gao, X. and D. Pan, *TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth.* Genes Dev, 2001. **15**(11): p. 1383-92.
94. Radimerski, T., et al., *Lethality of Drosophila lacking TSC tumor suppressor function rescued by reducing dS6K signaling.* Genes Dev, 2002. **16**(20): p. 2627-32.
95. Tee, A.R., et al., *Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling.* Proc Natl Acad Sci U S A, 2002. **99**(21): p. 13571-6.
96. Gao, X., et al., *Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling.* Nat Cell Biol, 2002. **4**(9): p. 699-704.
97. Zhang, Y., et al., *Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins.* Nat Cell Biol, 2003. **5**(6): p. 578-81.
98. Ma, L., et al., *Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis.* Cell, 2005. **121**(2): p. 179-93.
99. Tee, A.R., R. Anjum, and J. Blenis, *Inactivation of the tuberous sclerosis complex-1 and -2 gene products occurs by phosphoinositide 3-kinase/Akt-dependent and -independent phosphorylation of tuberin.* J Biol Chem, 2003. **278**(39): p. 37288-96.
100. El-Hashemite, N., et al., *Loss of Tsc1 or Tsc2 induces vascular endothelial growth factor production through mammalian target of rapamycin.* Cancer Res, 2003. **63**(17): p. 5173-7.
101. Guba, M., et al., *Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor.* Nat Med, 2002. **8**(2): p. 128-35.
102. Hudson, C.C., et al., *Regulation of hypoxia-inducible factor 1alpha expression and function by the mammalian target of rapamycin.* Mol Cell Biol, 2002. **22**(20): p. 7004-14.
103. Zhou, J. and B. Brune, *NO and transcriptional regulation: from signaling to death.* Toxicology, 2005. **208**(2): p. 223-33.
104. Ferrara, N., H.P. Gerber, and J. LeCouter, *The biology of VEGF and its receptors.* Nat Med, 2003. **9**(6): p. 669-76.

105. Nellist, M., et al., *Identification and characterization of the interaction between tuberin and 14-3-3zeta.* J Biol Chem, 2002. **277**(42): p. 39417-24.
106. Nellist, M., M.A. Goedbloed, and D.J. Halley, *Regulation of tuberous sclerosis complex (TSC) function by 14-3-3 proteins.* Biochem Soc Trans, 2003. **31**(Pt 3): p. 587-91.
107. Hengstschlager, M., et al., *Tuberous sclerosis genes regulate cellular 14-3-3 protein levels.* Biochem Biophys Res Commun, 2003. **312**(3): p. 676-83.
108. Li, Y., et al., *Regulation of TSC2 by 14-3-3 binding.* J Biol Chem, 2002. **277**(47): p. 44593-6.
109. Li, Y., et al., *The p38 and MK2 kinase cascade phosphorylates tuberin, the tuberous sclerosis 2 gene product, and enhances its interaction with 14-3-3.* J Biol Chem, 2003. **278**(16): p. 13663-71.
110. Liu, M.Y., et al., *14-3-3 interacts with the tumor suppressor tuberin at Akt phosphorylation site(s).* Cancer Res, 2002. **62**(22): p. 6475-80.
111. Shumway, S.D., Y. Li, and Y. Xiong, *14-3-3beta binds to and negatively regulates the tuberous sclerosis complex 2 (TSC2) tumor suppressor gene product, tuberin.* J Biol Chem, 2003. **278**(4): p. 2089-92.
112. Tzivion, G. and J. Avruch, *14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation.* J Biol Chem, 2002. **277**(5): p. 3061-4.
113. Brugarolas, J.B., et al., *TSC2 regulates VEGF through mTOR-dependent and -independent pathways.* Cancer Cell, 2003. **4**(2): p. 147-58.
114. Kenerson, H.L., et al., *Activated mammalian target of rapamycin pathway in the pathogenesis of tuberous sclerosis complex renal tumors.* Cancer Res, 2002. **62**(20): p. 5645-50.
115. Kenerson, H., T.A. Dundon, and R.S. Yeung, *Effects of rapamycin in the Eker rat model of tuberous sclerosis complex.* Pediatr Res, 2005. **57**(1): p. 67-75.
116. Mak, B.C., et al., *The tuberin-hamartin complex negatively regulates beta-catenin signaling activity.* J Biol Chem, 2003. **278**(8): p. 5947-51.
117. Lu, Z., et al., *Human papillomavirus 16 E6 oncoprotein interferences with insulin signaling pathway by binding to tuberin.* J Biol Chem, 2004. **279**(34): p. 35664-70.
118. Schwarz, E., et al., *Structure and transcription of human papillomavirus sequences in cervical carcinoma cells.* Nature, 1985. **314**(6006): p. 111-4.
119. Song, S., H.C. Pitot, and P.F. Lambert, *The human papillomavirus type 16 E6 gene alone is sufficient to induce carcinomas in transgenic animals.* J Virol, 1999. **73**(7): p. 5887-93.
120. Haddad, L.A., et al., *The TSC1 tumor suppressor hamartin interacts with neurofilament-L and possibly functions as a novel integrator of the neuronal cytoskeleton.* J Biol Chem, 2002. **277**(46): p. 44180-6.
121. Lariviere, R.C. and J.P. Julien, *Functions of intermediate filaments in neuronal development and disease.* J Neurobiol, 2004. **58**(1): p. 131-48.
122. Ramesh, V., *Aspects of tuberous sclerosis complex (TSC) protein function in the brain.* Biochem Soc Trans, 2003. **31**(Pt 3): p. 579-83.
123. Lamb, R.F., et al., *The TSC1 tumour suppressor hamartin regulates cell adhesion through ERM proteins and the GTPase Rho.* Nat Cell Biol, 2000. **2**(5): p. 281-7.

124. Castelo, L. and D.G. Jay, *Radixin is involved in lamellipodial stability during nerve growth cone motility.* Mol Biol Cell, 1999. **10**(5): p. 1511-20.
125. Paglini, G., et al., *Suppression of radixin and moesin alters growth cone morphology, motility, and process formation in primary cultured neurons.* J Cell Biol, 1998. **143**(2): p. 443-55.
126. Nellist, M., et al., *Phosphorylation and binding partner analysis of the TSC1-TSC2 complex.* Biochem Biophys Res Commun, 2005. **333**(3): p. 818-26.
127. Abraham, R.T. and G.J. Wiederrecht, *Immunopharmacology of rapamycin.* Annu Rev Immunol, 1996. **14**: p. 483-510.
128. Harris, T.E. and J.C. Lawrence, Jr., *TOR signaling.* Sci STKE, 2003. **2003**(212): p. re15.
129. Luo, H., et al., *Inhibition of in vitro immunoglobulin production by rapamycin.* Transplantation, 1992. **53**(5): p. 1071-6.
130. Thomson, A.W., et al., *Comparative effects of rapamycin, FK 506 and cyclosporine on antibody production, lymphocyte populations and immunoglobulin isotype switching in the rat.* Immunopharmacol Immunotoxicol, 1993. **15**(4): p. 355-69.
131. Kim, H.S., et al., *Effects of cyclosporine and rapamycin on immunoglobulin production by preactivated human B cells.* Clin Exp Immunol, 1994. **96**(3): p. 508-12.
132. Bjornsti, M.A. and P.J. Houghton, *Lost in translation: dysregulation of cap-dependent translation and cancer.* Cancer Cell, 2004. **5**(6): p. 519-23.
133. Bjornsti, M.A. and P.J. Houghton, *The TOR pathway: a target for cancer therapy.* Nat Rev Cancer, 2004. **4**(5): p. 335-48.
134. Sun, S.Y., et al., *Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition.* Cancer Res, 2005. **65**(16): p. 7052-8.
135. Manning, B.D., *Balancing Akt with S6K: implications for both metabolic diseases and tumorigenesis.* J Cell Biol, 2004. **167**(3): p. 399-403.
136. Harrington, L.S., G.M. Findlay, and R.F. Lamb, *Restraining PI3K: mTOR signalling goes back to the membrane.* Trends Biochem Sci, 2005. **30**(1): p. 35-42.
137. Johannessen, C.M., et al., *The NF1 tumor suppressor critically regulates TSC2 and mTOR.* Proc Natl Acad Sci U S A, 2005. **102**(24): p. 8573-8.
138. Li, H., et al., *Epidermal growth factor receptor signaling pathways are associated with tumorigenesis in the Nf1:p53 mouse tumor model.* Cancer Res, 2002. **62**(15): p. 4507-13.
139. Donovan, S., et al., *Hyperactivation of protein kinase B and ERK have discrete effects on survival, proliferation, and cytokine expression in Nf1-deficient myeloid cells.* Cancer Cell, 2002. **2**(6): p. 507-14.
140. Dasgupta, B., et al., *Proteomic analysis reveals hyperactivation of the mammalian target of rapamycin pathway in neurofibromatosis 1-associated human and mouse brain tumors.* Cancer Res, 2005. **65**(7): p. 2755-60.
141. Wienecke, R. *Effective treatment of Tuberous Sclerosis Complex with rapamycin.* in *TSC International Research Conference.* 2004. Cambridge, UK.
142. Bissler, J. *Rapamycin therapy of angiomyolipomas in patients with Tuberous Sclerosis Complex and Sporadic Lymphangioleiomyomatosis.* in *TSC International Research Conference.* 2004. Cambridge, UK.

143. Dabora, S. *Effective targeted therapy for TSC: update on preclinical studies and plans for a multi-center clinical trial for TSC and LAM*. in *TSC Internatioanl Research Conference*. 2004. Cambridge, UK.
144. Lee, L., et al., *Efficacy of a rapamycin analog (CCI-779) and IFN-gamma in tuberous sclerosis mouse models.* Genes Chromosomes Cancer, 2005. **42**(3): p. 213-27.
145. El-Hashemite, N., et al., *Perturbed IFN-gamma-Jak-signal transducers and activators of transcription signaling in tuberous sclerosis mouse models: synergistic effects of rapamycin-IFN-gamma treatment.* Cancer Res, 2004. **64**(10): p. 3436-43.

Summary

In this thesis, research into the autosomal dominant disorder Tuberous Sclerosis Complex (TSC) has been presented. TSC is characterized by hamartomas and hamartias in multiple organs and tissues, affecting 1 in 5800-10000 individuals. This disorder is caused by mutations in one of two tumor suppressor genes, the *TSC1* gene on chromosome 9q34 or the *TSC2* gene on chromosome 16p13. The major function of the protein products of the *TSC1* and *TSC2* genes, hamartin and tuberin respectively, is to form a complex that inhibits mammalian target of rapamycin (mTOR) dependent cell growth in response to various stimuli.

The diagnosis of TSC is based on clinical and pathological examinations, and several imaging methods. The criteria for the diagnosis have changed immensely over the years and have taken its present form in 1998. The severity of the disease depends on the organ or organs involved, and the number, size and the location of the lesions. Phenotypic variability, even within families, is well documented. Although the majority of the lesions observed are benign in nature, patients may suffer from serious medical complications related to the disease including epilepsy, mental retardation, autism, disfiguring skin lesions, hypertension, renal failure and pneumothorax.

The phenotypic expression of TSC is probably the product of the gene involved, the nature, type and effect of the primary mutation, the nature, type and effect of the second hit, modifying genes and environmental factors. In Chapter 2.a of this thesis, the largest cohort of TSC patients to date is presented. We showed that *TSC1* mutations compared to *TSC2* mutations, familial mutations compared to *de novo* mutations, and female gender compared to male gender are associated with milder TSC phenotypes. Furthermore, in the group of patients where we did not identify a mutation in either the *TSC1* or *TSC2* gene most clinical features occurred less frequently compared to the group of patients with mutations. In this cohort, we were able to determine pathogenic mutations in 85% of the definite TSC patients. The main reasons, why we could not identify a mutation in the remaining 15%, include technical difficulties related to mutation analysis, medium size deletions missed by both PCR-based and probe-related screening techniques, mutations within the promoter/enhancer or intronic regions, and somatic mosaicism.

In approximately two-thirds of the cases TSC is caused by a *de novo* mutation. Many mutations are unique to the families they are identified in and the majority of TSC patients have a mutation in the *TSC2* gene. A broad spectrum of mutations has been identified in both genes; however large rearrangements, missense mutations and in-frame deletions are very rare in the *TSC1* gene. A large deletion we characterized, involving the *TSC1* gene and two neighbouring genes, is described in Chapter 2.b. This is the first example of multi-gene deletion at the *TSC1* locus and indicates that large deletions at this locus could be more frequent than currently estimated.

In order to determine the distinct effects of single amino acid changes to tuberin on the function of the hamartin-tuberin complex, we developed and applied functional assays. Chapter 2.c shows that pathogenic mutations in the *TSC2* gene affect the function of the hamartin-tuberin complex. Clinical manifestations associated with codon 905 missense mutations in the *TSC2* gene and the

functional analyses of these mutations are presented in Chapter 2.d. The mild disease phenotype discussed in this chapter highlights the effect of the primary mutation on the disease phenotype, raises the possibility of non-penetrance, challenges the notion of extensive clinical variation, and shows how difficult establishing a definite diagnosis of TSC can be. In Chapter 2.e the use of functional assays as a diagnostic tool is described. In two families, the index cases who did not fulfill the clinical diagnostic criteria inherited two non-truncating changes, one from each parent. We used functional assays in order to characterize these changes and identified pathogenic mutations in both families. This chapter emphasizes the complementary role of functional assays in the interpretation of mutation analysis results.

Chapters 2.d and 2.e illustrate the occurrence of 'mild' or 'greyzone' mutations. Some mutations do not seem to disrupt hamartin-tuberin complex function completely and are associated with milder phenotypes that can even escape a clinical diagnosis of TSC. However, the clinical diagnostic criteria currently in use seem to be adequate in identifying the patients that require medical attention and changing these criteria is not suggested. For patients with mild phenotypes or only minor signs, longitudinal clinical studies will be necessary to determine the natural disease course and allow a more accurate assessment of the penetrance of TSC.

Finally, preliminary work on the regulation of the hamartin-tuberin complex in the mTOR signaling pathway is presented in the Appendix. The 5'-AMP-activated protein kinase (AMPK) has been shown to phosphorylate tuberin at two sites, thereby activating it. We disrupted these sites by introducing mutations to them and studied the effects with our functional assays. Possibly, the activation of the hamartin-tuberin complex by AMPK involves positioning it in the vicinity of its molecular target. These and other studies on the function and regulation of the hamartin-tuberin complex will be continued and extended in our laboratory. Such studies will be indispensable for a full understanding of the molecular pathogenesis of TSC and several other disorders and will hopefully increase the scope of therapeutic intervention.

The research presented in this thesis will help clinicians to give better and tailored genetic counseling in the families we studied, enable prenatal testing in families carrying mutations, may act as a guideline determining the nature of non-truncating changes in the *TSC2* gene and has shed some more light on the functions of the tuberin-hamartin complex.

Samenvatting

In dit proefschrift wordt onderzoek naar de autosomaal dominante aandoening Tubereuze Sclerose Complex (TSC) beschreven. TSC wordt gekenmerkt door de aanwezigheid van hamartoma's en hamartia's in meerdere organen en weefsels en treft 1 op 5800-10000 personen. Deze aandoening wordt veroorzaakt door mutaties in één van twee tumorsuppressorgenen, het *TSC1* gen op chromosoom 9q34 of het *TSC2* gen op chromosoom 16p13. De belangrijkste functie van de eiwitprodukten van de *TSC1* en *TSC2* genen, respectievelijk hamartine en tuberine, is het vormen van een eiwitcomplex dat de mTOR-afhankelijke celgroei remt in respons op verschillende stimuli. De diagnose TSC wordt gebaseerd op klinische en pathologische bevindingen en toepassing van verscheidene beeldvormende technieken. De criteria voor de diagnose zijn in de loop der jaren herhaaldelijk aangepast en hebben hun huidige vorm gekregen in 1998. De ernst van de ziekte hangt af van het orgaan of de organen die zijn aangedaan en het aantal, de grootte en de plaats van de lesies. TSC staat bekend om grote variabiliteit in de ziekteverschijnselen, zelfs binnen families. Alhoewel de meerderheid van de voorkomende lesies goedaardig is, kunnen patiënten lijden aan ernstige medische complicaties die verband houden met de ziekte zoals epilepsie, mentale retardatie, autisme, ontsieringen van de huid, hypertensie, nieraandoeningen en pneumothorax.

De fenotypische expressie van TSC is waarschijnlijk het resultaat van het betrokken gen, de aard, het soort en het effect van de primaire mutatie, de aard, het soort en het effect van de "second hit", andere genen, die mogelijk het ziektebeeld beïnvloeden, èn omgevingsfactoren. In hoofdstuk 2.a van dit proefschrift wordt het - tot nu toe - grootste cohort TSC patiënten beschreven. We laten hier zien dat *TSC1* mutaties in vergelijking tot *TSC2* mutaties, familiaire mutaties in vergelijking tot *de novo* mutaties en het vrouwelijk geslacht in vergelijking tot het mannelijk geslacht geassocieerd zijn met een milder fenotype. Verder valt op dat in de groep patiënten waarin we geen mutatie in één van de twee *TSC* genen hebben kunnen identificeren, de meeste klinische kenmerken minder vaak vóórkomen dan in de groep patiënten waarin we wel een mutatie hebben kunnen vinden. In dit cohort, hebben we bij 85% van de patiënten een mutatie kunnen detecteren. De belangrijkste oorzaken van het niet vinden van een mutatie in de resterende 15% zijn de technische problemen die gepaard gaan met mutatie analyse, middelgrote deleties die zowel bij probe gerelateerde onderzoekstechnieken als bij technieken die gebaseerd zijn op de PCR methode gemist worden, mutaties in het promoter gebied of in de intronen èn somatisch mozaïcisme.

In ongeveer twee-derde deel van de gevallen wordt TSC veroorzaakt door een *de novo* mutatie. Veel mutaties zijn uniek voor de families waarin ze geïdentificeerd worden en de meerderheid van de TSC patiënten heeft een mutatie in het *TSC2* gen. Een breed spectrum van mutaties is geïdentificeerd in beide genen; grote herrangschikkingen, missense mutaties en in-frame deleties komen echter zelden voor in het *TSC1* gen. Een grote deletie van het *TSC1* gen en twee aangrenzende genen die we hebben gekarakteriseerd, wordt beschreven in hoofdstuk 2.b. Dit is het eerste voorbeeld van een deletie van meerdere genen op het *TSC1* locus en geeft aan dat grote deleties op deze locus frequenter kunnen vóórkomen dan tot nu toe wordt aangenomen.
Om de verschillende effecten van afzonderlijke aminozuurveranderingen in tuberine op de functie van het hamartine-tuberine complex te kunnen bepalen, hebben we functionele testen ontwikkeld

en toegepast. In hoofdstuk 2.c is te zien dat pathogene mutaties in het *TSC2* gen de functie van het hamartine-tuberine complex aantasten. Klinische kenmerken die geassocieerd zijn met codon 905 missense mutaties in het *TSC2* gen en de functionele analyses van deze mutaties worden gepresenteerd in hoofdstuk 2.d. In de bespreking van het milde ziektebeeld in dit hoofdstuk komt de rol van de primaire mutatie in het tot stand komen van het fenotype aan de orde, wordt de mogelijkheid van non-penetrantie geopperd en worden vraagtekens gezet bij het begrip 'grote klinische variatie' (binnen één familie). Het in dit hoofdstuk beschreven onderzoek illustreert tevens hoe moeilijk het soms is om een definitieve diagnose TSC te stellen. In hoofdstuk 2.e wordt het gebruik van een functionele test als een diagnostisch middel beschreven. In twee families erfde de indexpatiënt twee "non-truncating" veranderingen, één van elke ouder. Dit type veranderingen is niet met zekerheid ziekte-veroorzakend. In beide gevallen voldeed de indexpatiënt niet aan de klinisch diagnostische criteria. Om de veranderingen te karakteriseren hebben we functionele testen gebruikt en in beide families hebben we pathogene mutaties geïdentificeerd. Dit hoofdstuk legt de nadruk op de aanvullende rol die functionele testen kunnen spelen bij de interpretatie van de resultaten van mutatie analyse.

De hoofdstukken 2.d en 2.e behandelen mutaties die als 'mild' bestempeld kunnen worden. Sommige mutaties lijken de functie van het hamartine-tuberine complex niet volledig te verstoren en zijn geassocieerd met een milder fenotype dat zelfs onvoldoende kan zijn voor het stellen van de klinische diagnose TSC. De huidige klinisch diagnostische criteria lijken echter adequaat om patiënten te identificeren die medische zorg nodig hebben en het veranderen van deze criteria wordt dan ook niet aangeraden. Voor patiënten met een mild fenotype of slechts enkele tekenen van de ziekte, zullen langlopende klinische onderzoeken nodig zijn om het natuurlijk verloop van de ziekte te kunnen bepalen en om een nauwkeuriger inschatting van de penetrantie van TSC mogelijk te kunnen maken.

Als laatste worden in de Appendix inleidende proeven in het onderzoek naar de regulatie van het hamartine-tuberine complex beschreven. Het 5'-AMP-geactiveerde eiwit-kinase (AMPK) blijkt tuberine op twee plaatsen te kunnen fosforyleren, waardoor tuberine geactiveerd wordt. We hebben deze fosforylerings-plaatsen weten te verstoren door mutaties aan te brengen en hebben het effect hiervan bestudeerd door middel van onze functionele testen. Mogelijk gaat de activering van het hamartine-tuberine complex door AMPK gepaard met het positioneren van het complex in de nabijheid van z'n "moleculaire target". Deze en andere studies naar de functie en regulatie van het hamartine-tuberine complex zullen in ons laboratorium worden voortgezet en uitgebreid. Zulke studies zijn noodzakelijk om de moleculaire pathogenese van TSC en verschillende andere aandoeningen te kunnen doorgronden en zullen hopelijk de mogelijkheden voor therapeutisch ingrijpen in de toekomst verruimen.

Het onderzoek dat in dit proefschrift wordt beschreven zal clinici in staat stellen beter en gerichter erfelijkheidsadvies te geven in de families die we hebben bestudeerd, het maakt prenataal onderzoek in families met een mutatie mogelijk, het kan fungeren als een leidraad om de aard van "non-truncating" veranderingen in het *TSC2* gen vast te stellen en het heeft bijgedragen aan de kennis van de functies van het hamartine-tuberine complex.

Özet

Bu tezde, otozomal dominant bir rahatsızlık olan Tüberöz Skleroz Kompleks (TSC) üzerine yapılan araştırmalar sunulmaktadır. TSC bir çok doku ve organda hamartomlar ve hamartialar ile karakterize olup 5800 ile 10000'de bir kişiyi etkilemektedir. Bu rahatsızlık iki tümör süpresör geninden birindeki mutasyondan ileri gelmektedir. *TSC1* geni kromozom 9q34'te, *TSC2* geni ise kromozom 16p13'te yer almaktadır. *TSC1* ve *TSC2* genlerinin protein ürünleri olan hamartin ve tüberin, mTOR'u inhibe eden bir kompleks oluşturmakta ve bu vesile ile hücre büyümesini düzenlemektedir.

TSC tanısı, klinik muayene ile patolojik ve görüntüleme tetkikleri sonuçları ile konulmaktadır. Tanı kriterleri yıllar içerisinde oldukça büyük bir değişimden geçmiş ve 1998 yılında şu anda kullanılmakta olan halini almıştır. Hastalığın ciddiyeti etkilenen organ ya da organlara, ve lezyonların sayı, ebat ve yerleşimine bağlıdır. Aynı aile içerisinde bile fenotipik farklılıklar görülebilir ve bunlar bir çok kere dokümente edilmiştir. Görülen lezyonların birçoğu benin özelliktedir. Buna rağmen hastalarda epilepsi, zeka geriliği, otizm, disfigüre eden deri lezyonları, hipertansiyon, böbrek yetmezliği ve pnömotoraks gibi ciddi tıbbi komplikasyonlar görülebilir.

TSC'nin fenotipik ekspresyonu muhtemelen hangi genin etkilendiği, primer mutasyonun doğası, tipi ve etkisi, ikinci mutasyonun doğası, tipi ve etkisi, hastalığı modifiye eden genler ve çevresel faktörler ile belirlenmektedir. Bölüm 2.a'da bugüne dek yayınlanmış en büyük TSC hasta kohortu sunulmaktadır. *TSC1* mutasyonlarının *TSC2* mutasyonlarına oranla, ailesel mutasyonların *de novo* mutasyonlara oranla ve dişi cinsiyetin erkek cinsiyete oranla daha hafif TSC fenotipleri ile ilişkili olduğunu gosterdik. Bunların yanısıra, birçok klinik özelliğin, *TSC1* ya da *TSC2* genlerinde mutasyon bulamadığımız hastalarda, mutasyon bulduğumuz hastalara oranla daha az sıklıkta ortaya çıktığını gösterdik. Bu kohortta kesin TSC olan hastaların %85'inde patojenik mutasyonu belirleyebildik. Geri kalan %15 hastada mutasyon bulamamamızın temel nedenleri mutasyon analizine dair teknik problemler, hem PCR'a dayalı hem de prob'lara dayalı mutasyon tarama teknikleri ile yakalanamayan orta büyüklükteki delesyonlar, promotor/enhansör ya da intronik bölgelerdeki mutasyonlar, ve somatik mozaisizmi içermektedir.

Vakaların yaklaşık üçte ikisi *de novo* bir mutasyondan kaynaklanmaktadır. Mutasyonların birçoğu özgün olup sadece tanımlandıkları ailelerde bulunmaktadır. TSC hastalarının büyük bir bölümünde mutasyon *TSC2* geninde yer almaktadır. Her iki gende de birçok farklı mutasyon tanımlanmıştır, ancak büyük rearanjmanlar, missens mutasyonlar ve in-frame delesyonlar TSC1 geninde nadiren görülmektedir. TSC1 genini ve komşuluğundaki 2 geni kapsayan büyük bir delesyon Bölüm 2.b'de anlatılmaktadır. Bu, *TSC1* lokusunda tanımlanmış ilk çoklu gen delesyonudur ve bu lokusta halihazırda tahmin edilenden daha sık büyük delesyon olabileceğine işaret etmektedir.

Laboratuvarımızda tüberin'deki amino asit değişikliklerinin hamartin-tüberin kompleksinin fonksiyonu üzerindeki etkilerini belirlemek için fonksiyonel testler geliştirip uyguladık. Bölüm 2.c'de *TSC2* genindeki patojenik mutasyonların hamartin-tüberin fonksiyonu üzerine olan etkileri gösterilmektedir. *TSC2* geninin 905 kodonundaki missens mutasyonlara ilişkin klinik bulgular

ve bu mutasyonların fonksiyonel analizleri Bölüm 2.d'de verilmektedir. Bu bölümde tartışılan hafif hastalık fenotipi, primer mutasyonun hastalık fenotipine olan etkisinin altını çizmekte, non-penetrans olasılığını ortaya koymakta, olağanüstü klinik varyasyon nosyonunu zorlamakta, ve TSC'de kesin tanı koymanın ne kadar güç olabileceğini ortaya koymaktadır. Bölüm 2.e'de ise fonksiyonel testlerin tanısal amaçlı kullanımı anlatılmaktadır. İki ailede, her bir ebeveyn birer non-truncating nükleotid değişiklikiğini, klinik tanısal kriterleri karşılamayan indeks vakalara kalıtmışlardır. Bu tip nükleotid değişiklikleri her zaman hastalığa yol açmamaktadırlar. Fonksiyonel testler kullanılarak bu değişikler karakterize edilmiş ve her iki ailede de patojenik mutasyonlar tanımlanmıştır. Bu bölüm fonksiyonel testlerin mutasyon analizi sonuçlarının yorumlanmasındaki katkısını vurgulamaktadır.

2.d ve 2.e bölümleri "hafif" ya da "gri zon" mutasyonlarının varlığını göstermektedir. Bazı mutasyonlar hamartin-tüberin komleksinin fonksiyonunu tamamen ortadan kaldırmıyor gibi görünmekte ve klinik TSC tanısından kaçabilecek kadar hafif fenotiplere yol açabilmektedir. Ancak şu anda kullanımda olan klinik tanısal kriterler tıbbi yardıma ihtiyacı olan hastaların tanımlanmasında yeterli gibi görünmekte ve bu kriterlerin değiştirilmesi tavsiye edilmemektedir. Hafif fenotipi olan hastalar ya da sadece minör bulguları olan hastalar için, hastalığın doğal klinik seyrinin belirlenmesi ve TSC'de penetransın doğru bir şekilde değerlendirilebilmesi longitüdinal klinik çalışmaların yapılmasını gerektirecektir.

Son olarak, Apendiks'te hamartin-tüberin kompleksinin regülasyonu üzerine yaptığımız öncül çalışmalar yer almaktadır. 5'-AMP tarafından aktive edilen protein kinazin (AMPK) tüberini iki noktada fosforile ettiği ve bu vasıtayla aktive ettiği gösterilmiştir. Bu noktalar mutasyonlar yoluyla ortadan kaldırılmış, ve etkileri fonksiyonel testlerle incelenmiştir. Muhtemelen, hamartin-tüberin kompleksinin AMPK tarafından aktivasyonu kompleksin moleküler hedefinin yakınına lokalize olması ile gerçekleşmektedir. Laboratuvarımızda hamartin-tüberin kompleksinin fonksiyonu ve regülasyonu üzerine çalışmalar devam edecek ve daha da genişletilecektir. Böyle çalışmalar TSC ve diğer bazı rahatsızlıkların moleküler patogenezlerinin tam anlaşılması için vazgeçilmez olup terapötik girişimleri olumlu yönde etkileyecektir.

Bu tezde sunulan araştırma klinisyenlerin incelenen ailelere daha iyi ve hastalara özgün genetik danışmanlık verebilmelerine yardımcı olacak, mutasyon taşıyan ailelerde prenatal test yapılabilmesini sağlayacak, *TSC2* genindeki non-truncating değişikliklerin doğasını belirmede bir kılavuz ödevi görebilecektir ve hamartin-tüberin kompleksinin fonksiyonları üzerine biraz daha ışık tutmuştur.

Acknowledgements

Four years and seven months have gone by, and I am finally trying to write the “dankwoord”. There are very many people I would like to thank for this accomplishment, and hopefully the ones I forget to mention or cannot mention here know who they are. Thank you all for making this the best, the most interesting, the most adventurous, the most leerzame years of my life.

I first need to thank Prof.dr. H. Galjaard. The postgraduate student position I occupied was his initiative. Thank you very much for this once-in-a-lifetime opportunity.

I would like to thank Prof.dr. J. Wladimiroff for giving me the extra time and the opportunity to finish the experiments, and my thesis.

My promoter Prof.dr. B.A. Oostra. Dear Ben, thank you very much for your continuous support and useful advice over the years, your critical reading of my thesis and the “gezellige” barbeques of course.

My co-promotor Dicky Halley. Beste Dicky, je bent meer dan mijn baas geworden. De afgelopen vier jaar, heb ik heel veel geleerd, en heel veel meegemaakt, en door echt moeilijke perioden gegaan. Jij hebt me altijd gesteund, met elke beslissing, met elke moeite. Dit proefschrift zou niet bestaan zonder jou. Dank je wel voor alles.

Dear Ans, thank you very much for all the “goedkeuringen” in the diagnostic lab, teaching me how to work in a diagnostic lab, and your valuable comments and advices. I will definitely miss coming to you to talk about almost everything.

Dear Mark, although we often had different ideas and ways, I learnt almost everything about proteins from you. Thank you for your patience, for your understanding, and your efforts during this period.

Dear Anneke, thank you for the useful discussions, and all your efforts to be able to get clinical information and your input on everything clinical.

I would like to thank the whole DNA diagnostic team, and in particular Peter (who taught me how to DGGE), Dennis (who had to listen to Dicky and me almost everyday), Erwin and Raoul (for all the sequencing), Lida (for all the Southerns) and Norma and Wilma of course. In addition many thanks to Pino, Cokkie, Bert and Annelies.

Although we had a very small TSC group, we frequently had students. Many thanks to all students, who made working in the lab more fun. In particular I would like to thank Brenda (who also became a good friend), Diana (for the breaks on 2nd(!) floor) and Christan (also for the breaks on 2nd(!) floor and later for all the sequencings).

I would like to thank to all of my lab mates, Laura, Rachel, Elly, Michael, Walter, Leontine and Marian, and also Surya and Stefano. In addition very many thanks to Violeta... You have been a very good friend over the years. Thank you very much for all the support, the listening ear (and kusjes aan Boris en groetjes aan Sava natuurlijk!). I also would like to thank all our neighbours on 9th floor. Special thanks go to Edwin (for the gezelligheid), Alessio (because he's funny) and Esther (the party maker). And all my colleagues in "ons kamer"... Alice, Merel, Mark, Marian, Violeta, Femke and Maria, thank you all for ruining my concentration, or actually you're welcome for my ruining your concentrations...

Very many thanks to Arnold, Prof.dr.de Zeeuw, and Prof.dr.Lindhout (en Ben natuurlijk) for their critical reading of my thesis. Your comments have been very useful and my thesis has become much better.

Jeannette, you've been the most helpful. Thank you very much for taking care of almost everything. I cannot think of a department of clinical genetics without you.

Dear Tom, thank you, thank you, thank you. I know I was very late, I know it was very difficult, and I know I've put you through a lot of stress. Thank you very much for all your efforts, and making this thesis look the way it looks now.

I also need to thank many collaborators. Senno, thank you for making the database and letting me use it. Bernard, you are doing a wonderful job with the TSC clinic, thank you for all the support. An Jansen, it has been a very difficult period, for you and me both, but finally it is gone, thanks for letting me be a part of it. Prof. Andermann, thanks for the hospitality and the collaboration. Elizabeth Thiele, thanks for the great offer. Marja Wessels, Marieke Baars and Charlotte Dommering are also thanked for all their efforts and inputs.

Peter Heutink, thank you very much for handing my CV to Dicky. If it wasn't for you I wouldn't have been where I am here today.

I would also like to thank all the TSC patients and families. STSN, and Hans Ploegmakers in particular, thank you very much for your support.

Sevgili Burcu, desteğin, arkadaşlığın, her türlü yardımın için teşekkür ediyorum. Hollanda'ya ilk geldiğimde kendimi tamamen bir yabancı gibi hissetmememi sana borçluyum. Sevgili Emine, aynı departmanda çalışan iki Türk olarak, sanırım konuşmalarımızla birçok kişiyi meraklandırdık. Arkadaşlığın, değerli tavsiyelerin, sürekli ilgin için çok teşekkürler. Darısı başına diyelim mi? Aysel, biliyorum sana karşı çok vefasız çıktım. Içli köfteler, bazalar, yemekler ve kahveler için sonsuz teşekkürler.

Yeşim'im, canım arkadaşım benim. Taaa Brüksel'de, iki saat uzakta olmana rağmen, her derdime, her kafa karışıklığıma (ama her mutluluğuma da çok şükür) yetiştin. Bu kadar yakında, insanın kardeş kadar yakın gördüğü birisinin bulunmasi bile inanılmaz bir destek. Her şey için binlerce

teşekkür, arabadan Brugge haftasonlarına, Brüksel haftasonlarından Zeeland gezmelerine, her gelişinde bir şeye(!) ihtiyacım var mı diye sormana kadar. Serkan'a da teşekkürler. Bodrum tatili hala akıllardan silinmiş değil. Ve Banu tabi. Her ikimiz de gurbetçi, her ikimiz de aynı zamanlarda buralara geldik. Birbirimizi çok kısa bir süredir tanımamıza rağmen, birbirimize inanılmaz destek olduk. Kahve falları, Sezen Aksu konserleri, ve hala geri veremediğim Türk filmlerinin VCD'leri için teşekkürler.

Sevgili Nurten Akarsu Hocam, eğer siz olmasaydınız, ben genetik sevdamı geride bırakmış, TUS'a girmiş, ve bir pediatrist olarak çıkmıştım sanırım. Bilmiyorum artık iyi mi yaptınız? Bu tez herkesten çok sizin eseriniz, güveniniz, ve desteğiniz. Teşekkürler. Genetik ve daha genel anlamda da biyolojiyle ilgilenmemi sağlayan lise biyoloji öğretmenim Serpil Üngör'e ve hem biyoloji öğretmenim hem de daha sonralari çok daha fazlası olan Ayşe Ihsan'a sonsuz tesekkürler.

En natuurlijk mijn paranimfen... Miriam, ik heb veel van je geleerd. Je bent echt een goede vriend geworden. Ik vond het erg gezellig samen met je te werken, drinken, eten, uitgaan. Bedankt voor alles.

En Bianca natuurlijk... Het is geen toeval dat je net voor de familie komt Piyango, je bent bijna familie. Ik voelde me ook deel van jouw familie, dankzij je schattige ouders, Ab en Lenie. Je bent de beste vriend(in) die ik ooit had, en ook zal hebben. Je hebt me altijd gesteund, altijd bij mij geweest. Goede tijden, slechte tijden, je was er. Tijdens romantische problemen, tijdens familie problemen, tijdens problemen over werk, tijdens depressies, tijdens moeilijke beslissingen, je was er. Je was ook daar tijdens heerlijke vakanties, mooie trouwerijen, wilde weekeinden. Ik kan niet aan een betere paranimf denken. Bedankt voor de verrukkelijke vier jaar (en de komende vijftig hopelijk).

Ve son olarak benim ben olmamı (üstelik sadece genetik açıdan değil) sağlayan aileme teşekkür etmek istiyorum. Sevgili anneciğim ve babacığım, nihayet dört buçuk sene bu doktora teziyle sonuçlandı. Her birimiz için sıkıntılı, yorucu, zor zamanlardı. Ama sonunda umuyorum ki her ikiniz de "küçük" oğlunuzla gurur duyuyorsunuz. Bizleri bugünlere getirirken katlandığınız her türlü sıkıntı, üzüntüye ve fedakarlığa değdi umuyorum. Bundan sonra da sizlere layık evlatlar olmak için çalişmaya devam edeceğiz. Ve sevgili ağabeyim ve "yenge"m. Insanın zor durumda kaldığı anda arayabileceği ve o an (bir hafta gecikmeyi saymazsak) yardıma yetişeceğini bildiği bir ağabeyinin olması inanılmaz bir ferahlık. Herşey için sonsuz teşekkürler. Bu benim yavrumdu, darısı sizlerin başına.

Özgür Soncak

Soncak

Curriculum Vitae

Date of Birth	June 2, 1976
Place of Birth	Elazığ, Türkiye

2001 – present	PhD research on Tuberous Sclerosis Complex at the department of Clinical Genetics, Erasmus Medical Center, Rotterdam, the Netherlands.
1994 – 2000	Faculty of Medicine, Hacettepe University, Ankara, Türkiye. Degree of Doctor in Medicine. Research projects on Medical Genetics and Public Health.
1991 – 1994	Science High School, Izmir Ozel Turk Lisesi, Izmir, Türkiye. Research project on Plant Biology.
1987 – 1991	Secondary School, Izmir Ozel Turk Lisesi, Izmir, Türkiye.

Relevant Publications

O. Sancak, M. Nellist, M. Goedbloed, P. Elfferich, C. Wouters, A. Maat-Kievit, B. Zonnenberg, S. Verhoef, D. Halley and A. van den Ouweland. Mutational analysis of the TSC1 and TSC2 genes in a diagnostic setting: genotype-phenotype correlations and comparison of diagnostic DNA techniques in Tuberous Sclerosis Complex. *Eur J Hum Genet*. Vol 13, 2005:731-741.

M. Nellist, O. Sancak, M. A. Goedbloed, M. V. Veghel-Plandsoen, A. Maat-Kievit, D. Lindhout, B. H. Eussen, A. De Klein, D. J. Halley and A. M. Ouweland. Large Deletion at the TSC1 Locus in a Family with Tuberous Sclerosis Complex. *Genet Test*. Vol 9, 2005:226-230.

M. Nellist, O. Sancak, M. A. Goedbloed, C. Rohe, D. van Netten, K. Mayer, A. Tucker-Williams, A. M. van den Ouweland and D. J. Halley. Distinct effects of single amino-acid changes to tuberin on the function of the tuberin-hamartin complex. *Eur J Hum Genet*. Vol 13, 2005:59-68.

A. Jansen, O. Sancak, MD. D'Agostino, A. Badhwar, P. Roberts, G. Gobbi, R. Wilkinson, D. Melanson, Tampieri, R. Koenekoop, M. Gans, A. Maat-Kievit, M. Goedbloed, A.M.W. van den Ouweland, M. Nellist, M. Pandolfo, M. McQueen, K. Sims, E. Thiele, F. Dubeau, F. Andermann, D.J. Kwiatkowski, D.J.J. Halley, E. Andermann. Mild Phenotype in Tuberous Sclerosis Patients with Codon 905 Missense Mutations in the Tsc2 Gene. Submitted for publication.

O. Sancak, M. Nellist, M. Goedbloed, M. Wessels, A. Maat-Kievit, M. Baars, C. Dommering, A. van den Ouweland, D. Halley. Functional Assays as a Diagnostic Tool in Tuberous Sclerosis Complex. Submitted for publication.

Useful website addresses

- Stichting Tubereuze Sclerosis Nederland (STSN): Dutch website for patients and families with TSC with detailed information on TSC in Dutch.
  ⇒ **http://www.stsn.nl**
- Tuberous Sclerosis International (TSI): The website of the worldwide organisation of Tuberous Sclerosis Associations with links to national patient organizations.
  ⇒ **http://www.stsn.nl/tsi/tsi.htm**
- Tuberous Sclerosis Alliance (TSA): The website of the patient organization in the United States of America with detailed information on TSC in English.
  ⇒ **http://www.tsalliance.org**
- Tuberous Sclerosis Project: The website of the research laboratory of Dr. David Kwiatkowsky with mutations and polymorphisms identified in the *TSC1* and *TSC2* genes, last updated in 1999.
  ⇒ **http://tsc-project.partners.org/index.htm**
- The Cardiff-Rotterdam Tuberous Sclerosis Mutation Database: Mutations identified in the *TSC1* and *TSC2* genes, last updated in 1998.
  ⇒ **http://archive.uwcm.ac.uk/uwcm/mg/tsc_db/**
- The Michelle Foundation: The website of a Dutch foundation dedicated to worldwide promotion and financial support for research into TSC.
  ⇒ **http://www.michellefoundation.org/index.htm**
- Erfocentrum: Dutch website with information on inheritance and inherited diseases.
  ⇒ **http://www.erfocentrum.nl/**
- Nederlandse Anthropogenetische Vereniging (NAV) – Vereniging Klinische Genetica Nederland (VKGN) – Vereniging Klinisch Genetische Laboratoriumdiagnostiek (VKGL): Dutch website with links to clinical genetic centers and diagnostic laboratories in the Netherlands and information for patients.
  ⇒ **http://www.nav-vkgn.nl/**
- Erasmus Medical Center, Department of Clinical Genetics
  ⇒ **http://www2.eur.nl/fgg/kgen/**
- Vereniging Samenwerkende Ouder- en Patiëntenorganisaties (VSOP): Website of the alliance of Dutch organizations of patient with inherited diseases.
  ⇒ **http://www.vsop.nl/**
- Ensembl: The website of a joint project between the European Molecular Biology Laboratory - European Bioinformatics Institute (EMBL – EBI) and Sanger Institute developing a software system which produces and maintains automatic annotation on selected eukaryotic genomes.
  ⇒ **http://www.ensembl.org/index.html**
- National Center for Biotechnology Information (NCBI): The website from the United States of America with resources on molecular biology, and links to sequence, protein and literature databases.
  ⇒ **http://www.ncbi.nlm.nih.gov**