A CLINICAL EVALUATION AND A GENETIC STUDY OF TUBEROUS SCLEROSIS COMPLEX IN A COHORT OF SRI LANKAN CHILDREN

BY

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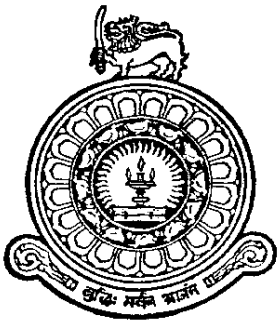
This research project was a collaboration of the following institutions.



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Declaration

I declare that the contents of this thesis are my own work, except for that detailed below, for which I would like to thank the following person:

- Ms. Ane- Marte Oye for sequencing and analisis of *TSC1* and *TSC2* genes.

CERTIFICATION

I certify that the contents of this dissertation are my own work and that I have acknowledged the sources where relevant.

……

Dr. L.D.Samudita Senaratne

This is to certify that the contents of this dissertation were supervised by the following supervisors:

…….	…….
Dr. Kaja Kristine Selmer	Dr. Jithangi Wanigasinghe
…….	……..
Dr Dulika Sumathipala	Prof. V.H.W. Dissanayake

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Abstract

Introduction: Tuberous Sclerosis Complex is a heterogeneous genetic disorder with autosomal dominant inheritance that affects almost all organ systems. The disorder is characterized by development of hamartomas in almost all organ systems of the body. Other characteristic features of Tuberous Sclerosis Complex are epilepsy, intellectual disabilities, autism spectrum disorders and organ failure. Tuberous Sclerosis Complex is no more considered a rare disorder, yet no cure has been found up to date. The knowledge has expanded to understand the tumor suppressor effects of Tuberous Sclerosis Complex genes on the mTOR pathway, seeking for new treatment options for the disease. This study is the first attempt to look into the Sri Lankan genetic composition with regard to this disease.

Objectives: The aims of this study were to describe the clinical phenotype of the children with Tuberous Sclerosis Complex in Sri Lanka. The *TSC1* and *TSC2* mutations were analyzed and a genotype – phenotype correlation of infantile spasms and polycystic kidney disease has been assessed.

Methods: In this study, we describe 44 children including all major ethnicities in Sri Lanka. Clinical history, physical examination findings and investigation findings such as brain MRI, CT and 2D echocardiogram were documented. Sanger sequencing was done for all the exons of *TSC1* and *TSC2* genes as the first line of detection of point mutations and small indels. In failing to detect the above, Multiplex Ligation dependent Probe Amplification was done to look for large deletions or insertions. These two methods provide a sensitive strategy in molecular diagnosis.

Results: The mutation detection rate was 85.7% in this study population. There were nine patients (21.5%) with mutations detected in *TSC1* gene and 27 patients (64.2%) with mutations in *TSC2* gene.

Conclusion: Though the clinical characteristics of Tuberous Sclerosis Complex in Sri Lankan children were similar to the previously described phenotype, there were 22 novel mutations in *TSC1* and *TSC2* genes reported in this study population. Among them, fourteen mutations had a high potential of being pathogenic. The presence of renal cysts (Grade 3, 4) was seen in five patients and mutations were solely found in the Gap domain of *TSC2* gene. A total of 19 patients had a history of infantile spasms. Their mutations were documented to be predominantly in the *TSC2* gene (16/19) in the peripheral gene region (13/16). There were six patients (14.3%) with Tuberous Sclerosis Complex phenotype, but where no mutations were found in coding regions of either gene, suggesting the need for further investigation.

1. Introduction and Background

1.1. History of Tuberous Sclerosis Complex

Tuberous Sclerosis Complex (TSC [MIM 191092 and MIM 191100]) is a heterogeneous genetic disorder with autosomal dominant inheritance that affects almost all organ systems [1]. The history of TSC started 150 years ago when the German pathologist Friedrich Daniel von Ricklinghausen first described the disease in 1862 with a case report of a new born who presented with multiple heart and brain tumors and died after a short while [2]. In 1880, Magloire Bourneville, a French neurologist first characterized and coined this disease as Tuberous Sclerosis with autopsy findings in a patient with intractable seizures, mental retardation and facial angiofibromas [3]. The term "Tuberous Sclerosis" referred specifically to multiple 'potato like' sclerotic lesions that were found in the cerebrum. Twenty eight years later, in 1908, the German neurologist Heinrich Vogt established three major clinical criteria to diagnose TSC which is known as the "Vogt'sTriad". This triad consists of epilepsy, mental retardation and adenoma sebaceum (facial angiofibromas). These were the first recorded crude diagnostic criteria for TSC [4]. Further exploration of the disease symptoms and signs, investigation into the cellular mechanisms that give rise to the disease and a quest for treatment has been a never ending process since then. Bourneville disease, Bourneville-Pringle disease, Tuberous Sclerosis (TS) and Tuberous Sclerosis Complex (TSC) are the different names used for the same disease [5]. At present this genetic disorder is commonly and more accurately termed Tuberous Sclerosis Complex in scientific literature in order to distinguish from Tourett's syndrome, an unrelated neurological disorder, as well as giving the idea of the complexity of the disease, its phenotypes and its variable expressivity. Although TSC was recognized to be a genetic disease 100 years ago, the underlying molecular etiology was revealed in early 1990s with the discovery of the two causative genes, *TSC1* and *TSC2* [6, 7].

Friedrich Daniel von Ricklinghausen (1833 – 1910)

Magloire Bourneville (1840 – 1909)

Fig1: Early investigators in TSC (Pictures from Wikipedia)

1.2. Genetics of Tuberous Sclerosis Complex

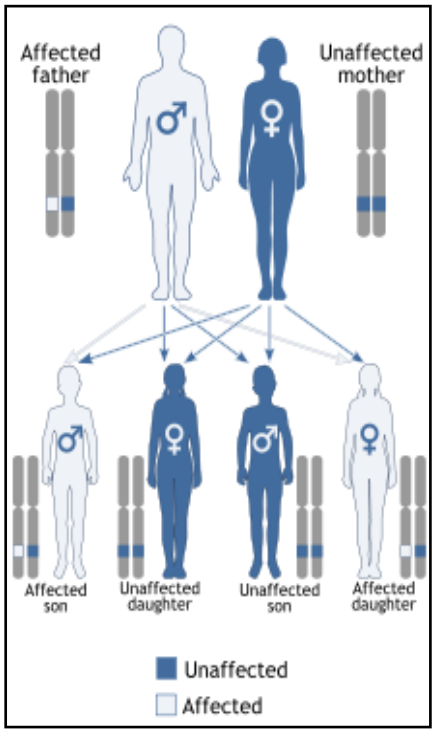


Fig2: Autosomal Dominant pattern of Inheritance (picture from Wikipedia)

Tuberous Sclerosis Complex is a neurocutaneous multisystem disorder which follows an autosomal dominant pattern of inheritance; therefore a pathogenic mutation in a single allele is sufficient to cause the disease [8]. It is also interesting to note that only 30% of the patients inherit the disease from either of their parents, while the remaining 70% is due to *de novo* mutations in *TSC1* or *TSC2* [9]. These *de novo* mutations are assumed to occur in the germ cells of either parent [10]. Theoretically, it can also occur as sporadic mutation in somatic cells of the early embryo. Germ line

mosaicism is also reported in families where parents without clinical manifestations have more than one child affected with TSC [8].

In autosomal dominant pattern of inheritance, the disease phenotype will be exhibited with a mutation in heterozygote state in an autosome (non sex chromosome). Therefore a parent with a mutant allele carries a risk of transmitting the disease allele to 50% of his or her offspring, irrespective of the gender. I.e. with each pregnancy there is a 50% risk of having an affected child. However, if a child is born of healthy parents who do not have detectable mutation in DNA from peripheral blood, the recurrence risk of having another child with TSC is considered to 1% to 2%. This small recurrence risk is due to germ line mosaicism. For the other relatives of the proband, depending on whether the parents carry the disease causing mutation and the degree of relationship, the risk varies [11]. Variable expressivity is the variable extent and intensity of phenotypic signs among people with a given genotype [12]. TSC is a very good example for variable expressivity. Variable expressivity results in great variation in clinical phenotype, ranging from single skin lesion in one member of the family to intractable seizures in another member [8]. Hence, expressivity is not determined by the specific gene mutation as different manifestations can be seen in members of the same family. Within the same family or among different families, there can be hundreds of phenotypic variants.

In some individuals disease manifestations are present at birth, but in others they might appear later in life. Age of onset does not seem to corelate with the severity of the disease [13]. The most common manifestations are the benign tumors in skin, brain, kidney, heart, eyes and lung. These may lead to organ dysfunction as the normal parenchyma is disrupted by tumor cells [2]. A range of neurobehavioral phenotypes with variable expression are associated with TSC and commonly seen are seizures, mental retardation, learning disabilities, attention deficit hyperactivity and autism [8]. TSC is typically a disease which

demonstrates pleiotropy, as this disease depicts a variety of apparently unrelated phenotypic features including skin hypopigmentation, multiple hamartomas in different organs, learning disability and multiple neurobehavioral phenotypes [14].

Penetrance is the frequency with which a genotype manifests itself in a given phenotype [12]. TSC is an autosomal dominant pleiotropic disorder with 100% or full penetrance [15]. Therefore, if the disease causing mutation is present in an individual, that will give rise to a disease phenotype even as a mild condition which can be clinically missed or undiagnosed. However, rare instances of non penetrance and reduced penetrance were reported in mid 1980s and early 1990s, but were later shown to be cases of germline mosaicism or occurence of two disease causing mutations [16-18].

In some disease conditions, the age of onset becomes lower in successive generations while the disease severity increases. This phenomenon is called the anticipation [12]. This phenomenon is commonly seen with genetically transmitted diseases with triplet repeat expansions. Although the parents with a milder phenotype transmitting the diseased allele to a more severely affected offspring is commonly noted in TSC, anticipation is not reported [11].

Great improvement in the understanding of the underlying pathomechanism was made with the discovery of the two disease causative genes *TSC2* in 1993 and *TSC1* in 1997 respectively. *TSC1* consists of a 3.4-kb coding region with 21 coding exons out of the total of 23 exons on 9q34 and *TSC2* consists of 5.4-kb coding region with 41 exons on 16p13.3 [19]. At present there are more than 1000 pathological mutations reported in *TSC1* and *TSC2* [8]. *TSC1* encodes the protein Hamartin and *TSC2* encodes the protein Tuberin. Hamartin and Tuberin interact directly to form a cytosolic complex and both *TSC1* and *TSC2* genes act as tumor suppressor genes [9]. According to previous studies, mutation detection rate in *TSC1*

and *TSC2* is approximately 85% to 89%. In the remaining 11% to 15% of the patients, no mutation is identified in these two genes, even if the clinical phenotype depicts TSC [2, 20]. Among all the mutations, *TSC1* mutations are found in 20% of the patients while the remaining 80% is accounted by *TSC2* mutations [21]. *TSC1* mutations as well as the familial *TSC2* mutations are less severe when compared to *de novo TSC2* mutations. Patients with no mutations in either genes are in some studies reported to have less severe phenotype than the patients with either *TSC1* or *TSC2* mutations [22]. Table 1 summarises the characteristics of the *TSC1* and *TSC2* genes [8, 11].

TABLE 1: Characteristics of the *TSC1* and *TSC2* genes[8, 11]

Characteristics	*TSC1*	*TSC2*
Localization	9q34	16p13.3
Structure	23 exons—8.6 kb transcript alternate splicing in the 5' UTR	41 exons—5.5 kb transcript exons 25, 26 and 31 alternatively spliced
Mutations	Small truncating mutations Missense mutations indels	Large deletions/rearrangements small truncating mutations missense mutations
Occurrence	10%-15% of sporadic cases 30% of familial cases	70% of sporadic cases 50% of familial cases
LOH in hamartomas	Rare	Frequent
Product	Hamartin	Tuberin
Subcellular localization	Cytoplasmic,? cortical	Cytoplasmic,? Golgi associated
Animal models	Knockout mice under development	Eker Rat Knockout mice, Drosophila (gigas)

UTR = Untranslated region. LOH = Loss of Heterozygosity. ? Probably.

1.3. Basic Disease Mechanism

The mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine- threonine kinase. This kinase is known to sense the environmental and cellular nutrition and energy status. Growth factors, nutrients and mitogens stimulate the activation of the two mTOR complexes, mTOR1 and mTOR2 to regulate diverse functions such as cell growth, development, proliferation, angiogenesis, memory, longevity, autophagy and innate as well as adaptive immune responses [23].

TSC1 and *TSC2* genes encode the proteins Hamartin and Tuberin, respectively. These two proteins form a complex in the cytoplasm and modulate cell functions such as cell growth, differentiation, migration and proliferation via mammalian target of rapamycin (mTOR) signaling cascade [24]. Figure 3 demonstrate the role of *TSC1-TSC2* complex in the mTOR signaling pathway [25].

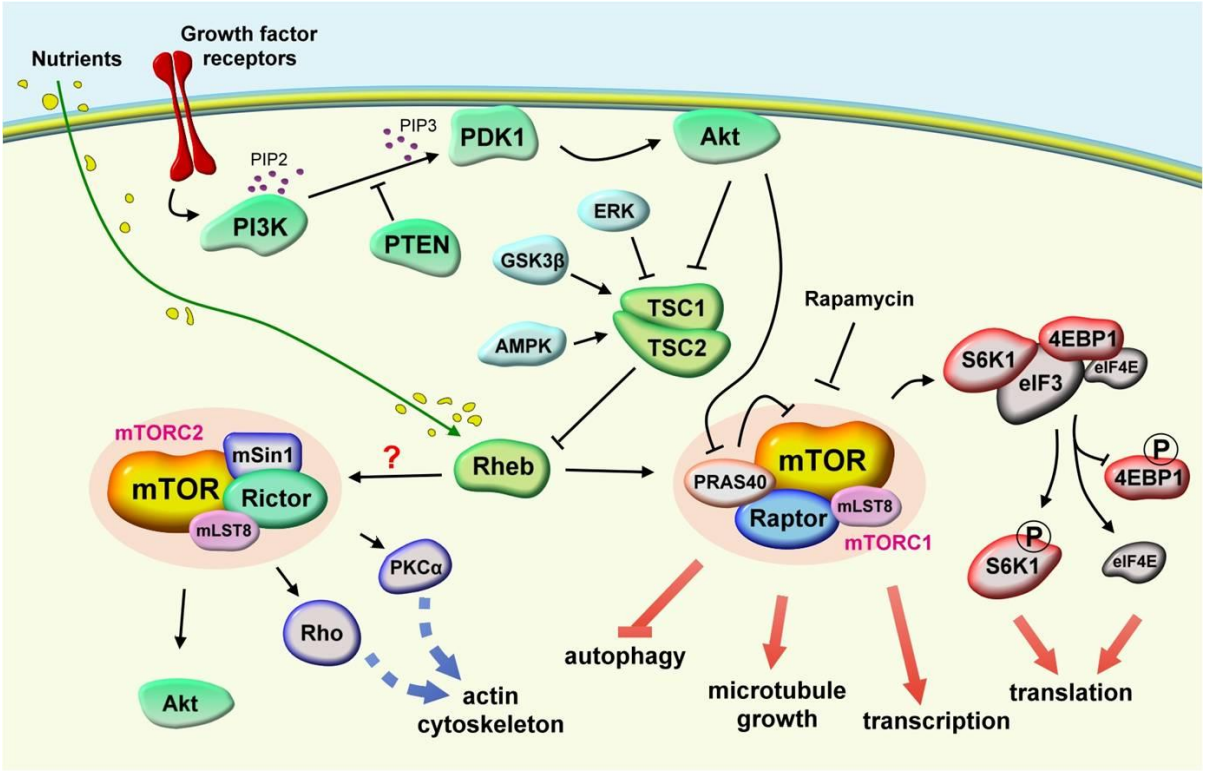


Fig 3 : Place of *TSC1-TSC2* complex in mTOR signaling pathway (with kind courtesy of Malgorzata Urbariska, MSc, International Institute of Molecular and Cell Biology, Warsaw, Poland)

The hamartin-tuberin protein complex does not act directly on mTOR. This complex acts as a tumor suppressor by activating GTP- binding protein Ras homolog enriched in brain (Rheb). This small G protein Rheb inhibits the mammalian target of rapamycin (mTOR) [26]. Co-factor of mTORC1 is the regulatory associated protein of mTOR (Raptor). Raptor activates the protein kinase domain of mTOR and thereby increases the mRNA transcription and protein synthesis. Mutation in either the *TSC1* or *TSC2* gene will inactivate the tumor suppressor action of the hamartin-tuberin complex, and thereby will also the inhibitory action of Rheb be. This will result in abnormal cell proliferation, and thereby formation of

nonmalignant tumors, the hamartomas. Rapamycin insensitive component of mTOR (Rictor) is the co-factor of mTORC2 complex which regulate protein synthesis in a manner different to mTORC1 and is unaffected by Rheb [27].

The molecular mechanisms in TSC reveal that the hamartomas can develop in almost all body systems by constitutive activation of the protein kinase mTOR due to haplo - insufficiency of either of the *TSC1* or *TSC2* gene [27]. The most commonly affected organ is the skin followed by the brain, kidney, heart and lung. Less commonly affected are the eye, liver, gut, bone, mouth and teeth.

1.4. Affected organ systems

Skin: Skin is the most commonly affected organ in TSC and more than 96% of the patients are found to have skin manifestations [28]. Table 2 summaries the common skin manifestations in TSC [2, 3, 28].

TABLE 2: Summary of skin manifestations in TSC

Skin manifestation	Prevalence	Age of presentation	Diagnostic importance	Comments
Hypopigmented patches	>90%	Birth, infancy, childhood	Major diagnostic criterion	Presenting sign in TSC. Number increases with age
Facial angiofibromas (FA)	75%	2-5 years	Major diagnostic criterion, when =/> 3	Presentation in adulthood taken as minor diagnostic criterion after confirmation with skin biopsy
Fibrous cephalic plaques	25%	2-5 years	Major diagnostic criterion, when =/>3	Most specific skin lesion. Histologically similar to FAs. Paired with FA in diagnosis
Shagreen patches	50%	1st decade of life	Major diagnostic criterion	Large plaques on lower back with bumpy or orange peel appearance. Nearly pathognomonic to TSC
Ungual fibromas	< 20% (overall) 80% (old population)	2nd decade of life	Major diagnostic criterion, when =/>2.	>80% noted in older adults
Confetti skin lesions	3% (paediatric population) 58% (overall)	1st decade of life	Minor diagnostic criterion	1-3mm hypopigmented maculae. Commonly scattered over arms and legs
Café au lait patches	30%	Birth, infancy, childhood	Not a diagnostic criterion	Hyperpigmented maculae.

=/>: equal or more

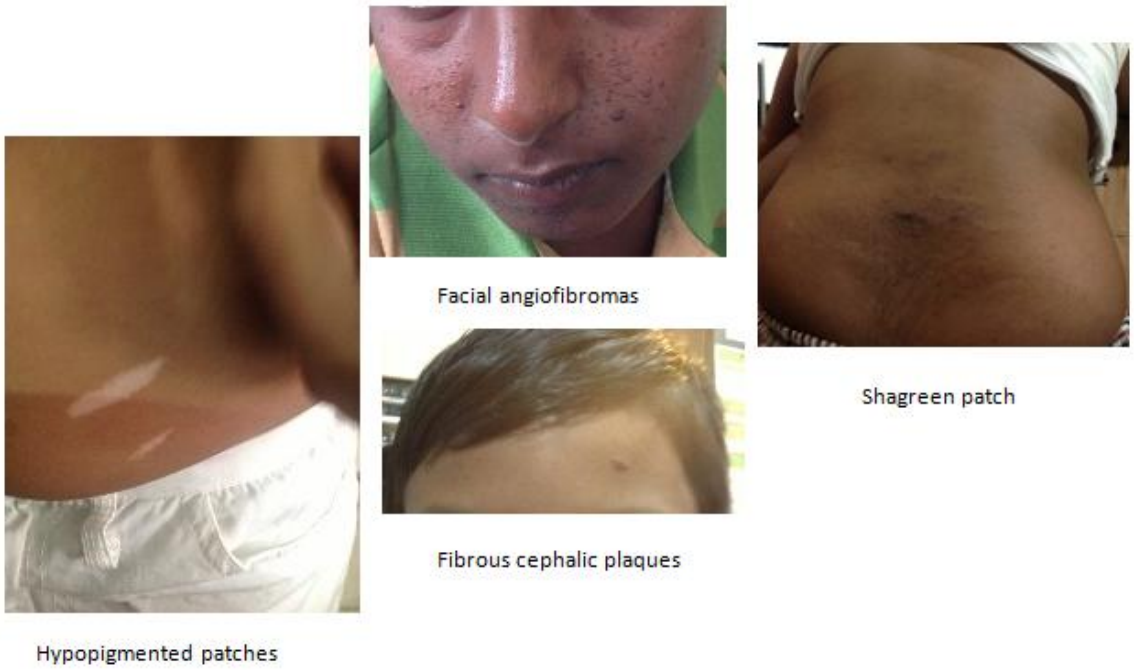


Fig 4: Skin manifestations in TSC

Brain: The greatest morbidity and mortality in TSC is associated with brain manifestations. TSC associated structural abnormalities of the brain, epilepsy and neuropsychiatric disorders are the three major phenotypes associated with the central nervous system [2].

Cortical tubers, a form of focal cortical dysplasias, are the hallmark of TSC. Histologically they consist of giant neurons and astrocytes [27]. Cortical dysplasia are congenital abnormalities where a group of neurons fail to migrate to the expected area of the brain during embryonic development [2]. Cortical tubers are found in 90% of the patients with TSC by neuro imaging such as Magnetic Resonance Image (MRI). Other forms of cortical dysplasia such as cerebral white matter radial migration lines can be found together with cortical tubers in TSC and when there are two or more areas of cortical dysplasia, it is considered as a major diagnostic criterion. Cortical dysplasias are commonly associated with intractable epilepsy and learning difficulties in TSC patients [2].

Other structural manifestations of the brain are the subependymal nodules (SEN) and the subependymal giant cell astrocytomas (SEGA). SEN can be detected in 80% of the patients with TSC prenatally or at birth. They are non-malignant tumors with slow progression which develop along the wall of the ependymal lining of lateral and third ventricles [2].

SEGAs are histologically similar to SEN and incidence in TSC is 5-15%. They can be present prenatally or at birth, but are much more likely to arise during childhood or adolescence. They seldom occur after 20 years. It is accepted that SEGA arises from SEN near the foramen of Monro. Although they are slow growing they can lead to critical situations where urgent surgical resection is necessary as they might obstruct foramen of Monro, leading to high intracranial pressure. Both SEN and SEGA may progressively calcify and stop growing [2, 27].

According to recent studies, 90% of the patients with TSC have epilepsy [29]. Common types of seizures are the infantile spasms (IS), which occur in 30 – 40% of the patients, and partial seizures. Partial seizures can progress in to generalized tonic clonic seizures due to rapid bilateral synchronization [27]. Several studies show early onset of infantile spasms is associated with poorer prognosis with developmental delay, autism and intractable partial epilepsies later in life [27]. In TSC most of the patients with IS do not depict the typical electro encephalogram (EEG) pattern with hypsarrythmia and therefore the diagnosis of IS in TSC patients can be masked [29]. Hence it is advisable to treat IS in TSC purely on clinical grounds independent of EEG. For patients with medically intractable partial epilepsies, a remedy is soughed with methods such as the ketogenic diet, vagal nerve stimulation and respective epilepsy surgery [27].

Among the patients with TSC, 50-60% has neuropsychiatric disorders such as autism, mental retardation, attention deficit hyperactivity, learning disabilities and other behavioural problems.

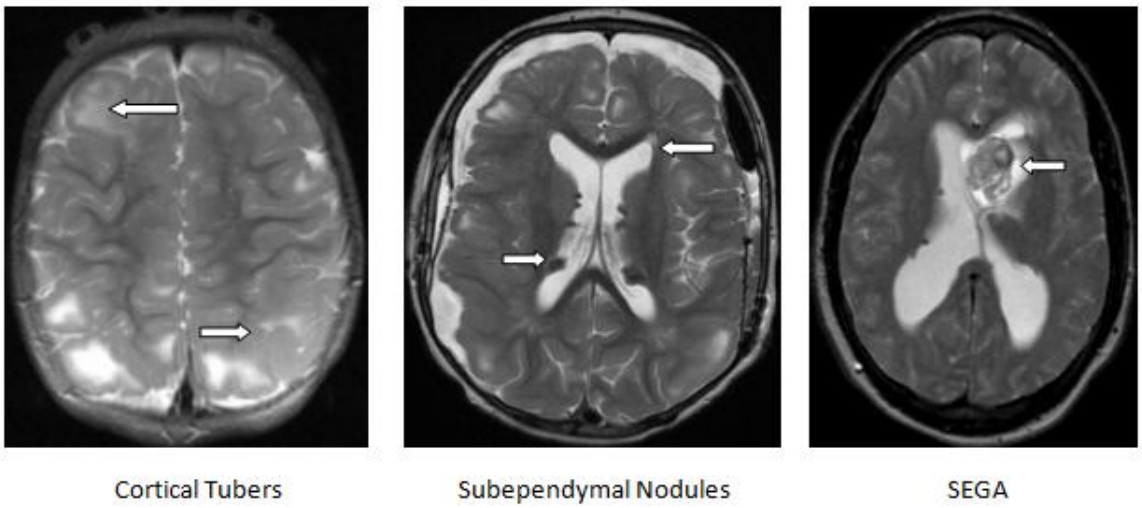
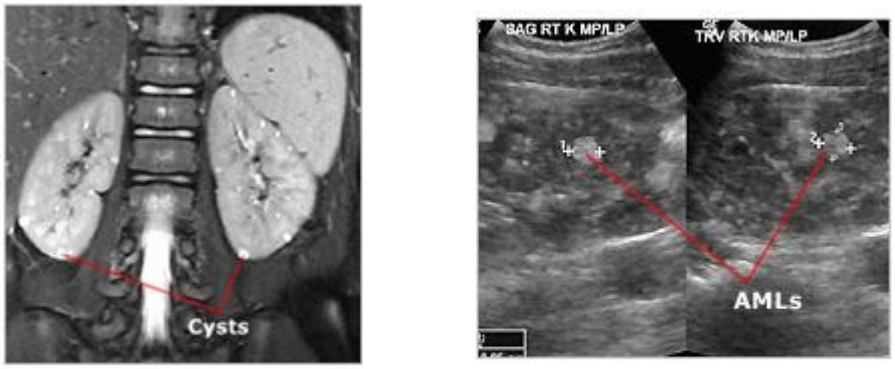


Fig 5: Three major diagnostic criteria in TSC that can be noted with neuro imaging techniques (With kind complement of Dr. Jonathan Roth, Department of Pediatric Neurosurgery, Dana Children's Hospital, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel.)

Kidney: Renal complications are the second most common cause of morbidity and mortality [30] and renal angiomyolipomas are present in 75% - 80% of the patients with TSC [3]. Angiomyolipomas are benign tumors composed of thickened blood vessels, adipose tissue and smooth muscle and they are relatively specific to TSC. Two or more angiomyolipomas in any organ is considered as a major diagnostic criterion in TSC and the most common organ affected is the kidney [2]. The renal manifestations in TSC begin in infancy and progress with age. The rapid growth of angiomyolipomas are noted during childhood and adolescence and slow down towards adulthood [27]. Females are more commonly affected, with a female to male ratio of 4:1 [3]. The smaller angiomyolipomas are said to be asymptomatic, but when the lesions exceed 4cm in size, they can give rise to life threatening complications such as haemorrhage, acute renal failure or shock [3]. Lesions greater than 4cm are usually found in post pubertal patients [27]. The second most common lesions found in the kidney are the renal cysts which are present in 45% of the patients with TSC [27]. The combination of renal cysts and angiomyolipomas is characteristic of TSC [31]. Renal micro-cysts can be present in patients with both *TSC1* and *TSC2* mutations and appear earlier in life than the angiomyolipomas [3]. Severe polycystic kidneys appears only in

patients with *TSC2* gene deletions that gives rise to contagious gene syndrome involving the *PKD1* gene deletions [32]. *PKD1* gene is transcribed in the opposite direction to *TSC2* gene and is only 48 base pairs away from *TSC2* gene on chromosome16.3 [2]. This contagious deletion is reported in 2% of the patients with TSC [27] and gives rise to TSC phenotype combined with a more aggressive autosomal dominant polycystic kidney disease(ADPKD) presenting during infancy and childhood [2].



Renal MRI scan with bilateral renal cysts

Renal Ultrasound Scan right kidney with angiomyolipomas

Fig 6: Renal involvements in TSC (with permission from Herscot Center for TSC at Massachusetts General Hospital, Boston, Massachusetts, USA)

Lung: Lymhangioliomyomatosis (LAM) is a late presentation in TSC and appear in 2nd or 3rd decade of life nearly exclusively in females with TSC. However, some male TSC cases have been reported to have LAM findings in biopsy material [27]. LAM is the most common lung manifestation in TSC and is observed in 30-40% of the female patients in child bearing age, though only 1% of them are found to be symptomatic [2, 3]. Histologically, LAM is diffuse infiltration of lung tissue with atypical smooth muscle cells. Gradual replacement with cysts in pulmonary parenchyma occur at a later stage [27]. As LAM affects almost exclusively females, there is strong evidence of a hormonal component being involved in the pathomechanism [32]. LAM is the third most common cause of mortality in TSC and is a

major diagnostic criterion in TSC. Presence of LAM without TSC is classified as sporadic LAM. Angiomyolipoma is a finding in one third of the patients with sporadic LAM. Therefore when both LAM and angiomyolipomas are present in a patient with TSC, it is considered as one major diagnostic criterion rather than two [2].

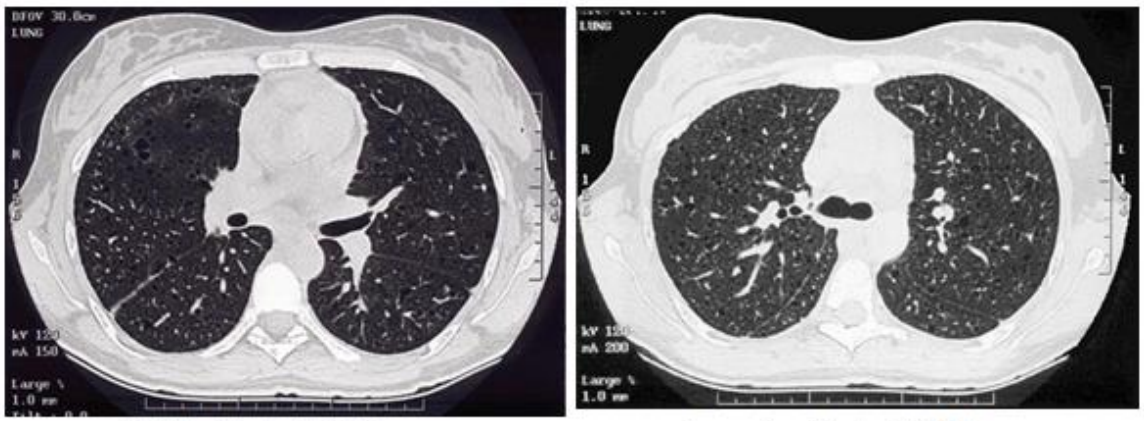


Fig 7: MRI scans of lungs affected with LAM. (The images are from the National Heart, Lung, and Blood Institute (NHLBI) at the National Institutes of Health (NIH) in Bethesda, MD, USA.)

Heart: Cardiac rhabdomyomas (CR) are present in approximately 50% of all patients with TSC and are usually congenital as they develop between 22 to 26 weeks of gestation [3]. CRs are mostly asymptomatic and can be a finding in routine checkups. It might also present as a cardiac murmur, a rhythm disturbance, the Wolff-Parkinson-White syndrome or as cyanosis. In general CRs are multiple, well circumscribed hyperechogenic masses frequently located in interventricular septum or the free wall of the heart [33]. These solid tumors regress spontaneously and disappear completely or partially within infancy to early childhood in more than 50% of all cases [34]. The once that cause serious cardiovascular complications such as outflow tract obstruction, reduced cardiac function or rhythm disturbances may need surgical or medical intervention [35].

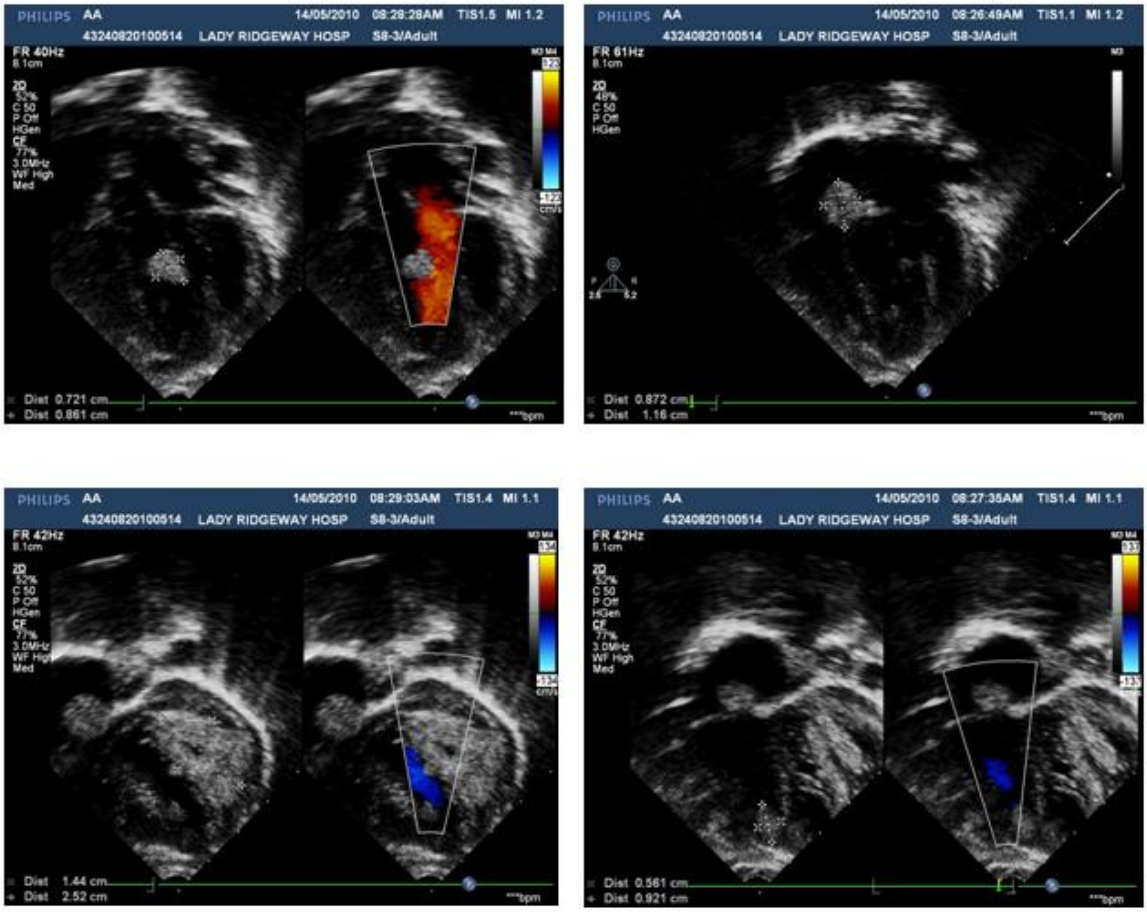


Fig 8: 2D Echocardiogram of Multiple CRs in a patient with TSC (With kind complement of Dr. Duminda Samarasinghe, Consultant Paediatric Cardiologist- Lady Ridgeway Hospital for Children, Colombo, Sri Lanka)

Eye: Astrocytic hamartoma (AH) is a characteristic retinal finding and is also considered as a major criterion of diagnosing TSC. They commonly present as multiple lesions, and are histologically similar to cortical tubers in the brain [2]. As a AH usually does not cause visual impairment, it rarely require intervention and is a good marker of TSC. Eye manifestations are found in approximately 35% of the patients with TSC. Retinal Achromic Patches(AP) might also be observed but, are less common and are considered as a minor criterion [36].

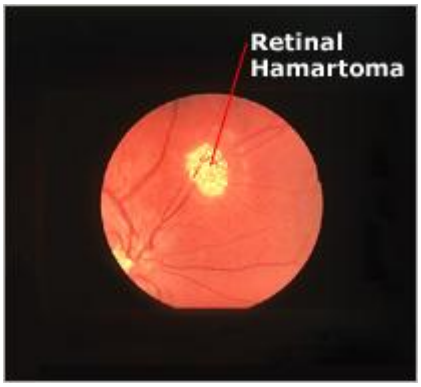


Fig 9: Image of a retinal hamartoma in a patient affected with TSC (with permission from Herscot Center for TSC at Massachusetts General Hospital, Boston, Massachusetts, USA)

Mouth and Teeth*:* Intra oral fibromas (when there are two or more) and dental enamel pits (when there are three or more) are considered as minor diagnostic criteria in TSC [2]. Intra oral fibromas are said to be present in 20 – 50% of the patients with TSC, but with a higher frequency in adulthood [28, 37]. Dental enamel pits are significantly more common among adult TSC patients when compared to the normal healthy adults [38]. Prevalence among the paediatric population is not known.

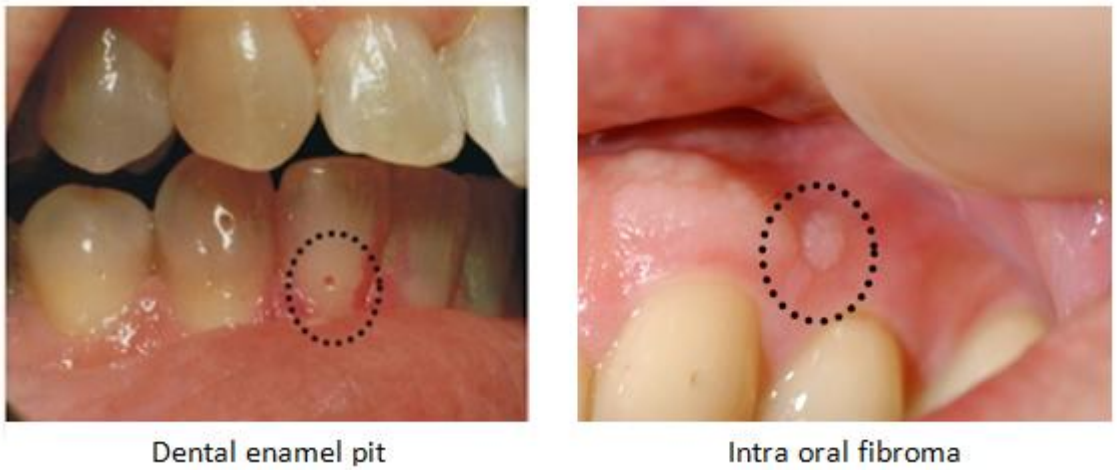


Fig 10: Minor diagnostic criteria associated with mouth & teeth. (These photos are courtesy of Greg Mlynarcyzk, DDS, a member of the TS Alliance Professional Advisory Board.)

Other clinical, but less frequent characteristics of TSC include lesions in bone, in gastrointestinal system and in endocrine organs [2]. Bone cysts are less frequently seen in TSC and are not specific to the disease. They are rarely identified when the other features of TSC are absent, and are therefore no more considered as a diagnostic criterion. The same is true for the hamartomatous rectal polyps. Liver angiomiolipomas are acknowledged as nonrenal angiomyolipomas [2].

1.5. Clinical Aspects of TSC

TSC is a neurocutaneous disorder that affects almost all body systems. There are many neurocognitive and neurobehavioral phenotypes also associated with TSC. Therefore a multi disciplinary approach is required in treating patients with TSC. This multi disciplinary effort includes the neurologist, dermatologist, nephrologists, ophthalmologist, cardiologist, pulmonologist, psychiatrist, radiologist, dentist and surgeons of various disciplines. Geneticists and genetic counselors also play a major role with the discovery of genetic mutations and alterations in molecular mechanisms related to disease causation. At the 2012 International Tuberous Sclerosis Consensus Conference, the diagnostic criteria for TSC were updated. Clinical features of TSC continues to be the principal means of diagnosis while changes compared to 1998 criteria are the inclusion of genetic test results and reducing diagnostic classes from three (possible, probable, definite) to two (possible and definite) [2]. Table 3 shows the updated diagnostic criteria for TSC in 2012 at the International Tuberous Sclerosis Consensus Conference. It mainly consists of 2 parts, genetic diagnostic criteria and clinical diagnostic criteria. Clinical criteria are again subdivided into major and minor criteria. It emphasizes that diagnosing a pathological mutation in either *TSC1* or *TSC2* genes is sufficient to diagnose TSC [2].

Testing for mutations in *TSC1* and *TSC2* genes is not only important in diagnosis of TSC, but it is also important in family screening, risk assessment and counseling according to the genetic makeup of each and every family member.

TABLE 3: Updated diagnostic criteria for tuberous sclerosis complex 2012 [2].

A. Genetic diagnostic criteria

The identification of either a TSC1 or TSC2 pathogenic mutation in DNA from normal tissue is sufficient to make a definite diagnosis of tuberous sclerosis complex (TSC).
A pathogenic mutation is defined as a mutation that clearly inactivates the function of the TSC1 or TSC2 proteins (e.g., out-of-frame indel or nonsense mutation), prevents protein synthesis (e.g., large genomic deletion), or is a missense mutation whose effect on protein function has been established by functional assessment (www.lovd.nl/TSC1, www.lovd/TSC2, and Hoogeveen-Westerveld et al., 2012 and 2013).
Other TSC1 or TSC2 variants whose effect on function is less certain do not meet these criteria, and are not sufficient to make a definite diagnosis of TSC. Note that 10% to 25% of TSC patients have no mutation identified by conventional genetic testing, and a normal result does not exclude TSC, or have any effect on the use of clinical diagnostic criteria to diagnose TSC.

B. Clinical diagnostic criteria

Major features

1. Hypomelanotic macules (≥3, at least 5-mm diameter)
2. Angiofibromas (≥3) or fibrous cephalic plaque
3. Ungual fibromas (≥2)
4. Shagreen patch
5. Multiple retinal hamartomas
6. Cortical dysplasias*
7. Subependymal nodules
8. Subependymal giant cell astrocytoma
9. Cardiac rhabdomyoma
10. Lymphangioleiomyomatosis (LAM) y
11. Angiomyolipomas (≥2)y

Minor features

1. "Confetti" skin lesions
2. Dental enamel pits (>3)
3. Intraoral fibromas (>2)
4. Retinal achromic patch
5. Multiple renal cysts
6. Nonrenal hamartomas

Definite diagnosis: Two major features or one major feature with ≥2 minor features
Possible diagnosis: Either one major feature or ≥2 minor features
* Includes tubers and cerebral white matter radial migration lines.
y A combination of the two major clinical features (LAM and angiomyolipomas) without other features does not meet criteria for a definite diagnosis

Once the diagnosis of TSC has been established according to the above mentioned guidelines, it is important to manage and follow up the patients according to the standard protocols. Due to the diverse and varied presentation and progression of disease manifestations management plan will be exclusive to each and every patient. Depending on the availability of resources and the other practicalities management practices are highly variable among region and

country [1]. Table 4 & 5 shows the standardized approach for clinical management of patients with TSC which was reviewed and recommended at the 2012 International Tuberous Sclerosis Consensus Conference. These recommendations are applicable for the newly diagnosed patients as well as the patients with established diagnosis [1].

TABLE 4: Management recommendations for the newly diagnosed and suspected TSC [1].

Organ System or Specialty Area	Recommendation
Genetics	• Obtain three-generation family history to assess for additional family members at risk of TSC • Offer genetic testing for family counseling or when TSC diagnosis is in question but cannot be clinically confirmed
Brain	• Perform magnetic resonance imaging (MRI) of the brain to assess for the presence of tubers, subependymal nodules (SEN), migrational defects, and subependymal giant cell astrocytoma (SEGA) • Evaluate for TSC-associated neuropsychiatric disorder (TAND) • During infancy, educate parents to recognize infantile spasms, even if none have occurred at time of first diagnosis • Obtain baseline routine electroencephalogram (EEG). If abnormal, especially if features of TAND are also present, follow-up with a 24-hr video EEG to assess for subclinical seizure activity
Kidney	• Obtain MRI of the abdomen to assess for the presence of angiomyolipoma and renal cysts • Screen for hypertension by obtaining an accurate blood pressure • Evaluate renal function by determination of glomerular filtration rate (GFR)
Lung	• Perform baseline pulmonary function testing (pulmonary function testing and 6-minute walk test) and high-resolution chest computed tomography (HRCT), even if asymptomatic, in patients at risk of developing lymphangioleiomyomatosis (LAM), typically females 18 years or older. Adult males, if symptomatic, should also undergo testing • Provide counsel on smoking risks and estrogen use in adolescent and adult females
Skin	• Perform a detailed clinical dermatologic inspection/exam
Teeth	• Perform a detailed clinical dental inspection/exam
Heart	• Consider fetal echocardiography to detect individuals with high risk of heart failure after delivery when rhabdomyomas are identified via prenatal ultrasound • Obtain an echocardiogram in pediatric patients, especially if younger than 3 yr of age • Obtain an electrocardiogram (ECG) in all ages to assess for underlying conduction defects
Eye	• Perform a complete ophthalmologic evaluation, including dilated funduscopy, to assess for retinal lesions and visual field deficits

TABLE 5: Management recommendations for patients already diagnosed with tuberous sclerosis complex (TSC) [1].

Organ System or Specialty Area	Recommendation
Genetics	• Offer genetic testing and family counseling, if not done previously, in individuals of reproductive age or newly considering having children
Brain	• Obtain magnetic resonance imaging (MRI) of the brain every 1-3 yr in asymptomatic TSC patients younger than age 25 yr to monitor for new occurrence of subependymal giant cell astrocytoma (SEGA). Patients with large or growing SEGA, or with SEGA causing ventricular enlargement but yet are still asymptomatic, should undergo MRI scans more frequently and the patients and their families should be educated regarding the potential of new symptoms. Patients with asymptomatic SEGA in childhood should continue to be imaged periodically as adults to ensure there is no growth. • Surgical resection should be performed for acutely symptomatic SEGA. Cerebral spinal fluid diversion (shunt) may also be necessary. Either surgical resection or medical treatment with mammalian target of rapamycin complex (mTOR) inhibitors may be used for growing but otherwise asymptomatic SEGA. In determining the best treatment option, discussion of the complication risks, adverse effects, cost, length of treatment, and potential impact on TSC-associated comorbidities should be included in the decision-making process. • Perform screening for TSC-associated neuropsychiatric disorders (TAND) features at least annually at each clinical visit. Perform comprehensive formal evaluation for TAND at key developmental time points: infancy (0-3 yr), preschool (3-6 yr), pre-middle school (6-9 yr), adolescence (12-16 yr), early adulthood (18-25 yr), and as needed thereafter. Management strategies should be based on the TAND profile of each patient and should be based on evidence-based good practice guidelines/practice parameters for individual disorders (e.g., autism spectrum disorder, attention deficit hyperactivity disorder, anxiety disorder). Always consider the need for an individual educational program (IEP). Sudden change in behavior should prompt medical/clinical evaluation to look at potential medical causes (e.g., SEGA, seizures, renal disease). • Obtain routine electroencephalograph (EEG) in individuals with known or suspected seizure activity. The frequency of routine EEG should be determined by clinical need rather than a specific defined interval. Prolonged video EEG, 24 hr or longer, is appropriate when seizure occurrence is unclear or when unexplained sleep, behavioral changes, or other alteration in cognitive or neurological function is present • Vigabatrin is the recommended first-line therapy for infantile spasms. Adrenocorticotropin hormone (ACTH) can be used if treatment with vigabatrin is unsuccessful. Anticonvulsant therapy of other seizure types in TSC should generally follow that of other epilepsies. Epilepsy surgery should be considered for medically refractory TSC patients, but special consideration should be given to children at younger ages experiencing neurological regression and is best if performed at epilepsy centers with experience and expertise in TSC.
Kidney	• Obtain MRI of the abdomen to assess for the progression of angiomyolipoma and renal cystic disease every 1-3 yr throughout the lifetime of the patient.

	• Assess renal function (including determination of glomerular filtration rate [GFR]) and blood pressure at least annually. • Embolization followed by corticosteroids is first-line therapy for angiomyolipoma presenting with acute hemorrhage. Nephrectomy is to be avoided. For asymptomatic, growing angiomyolipoma measuring larger than 3 cm in diameter, treatment with an mTOR inhibitor is the recommended first-line therapy. Selective embolization or kidney-sparing resection are acceptable second-line therapy for asymptomatic angiomyolipoma.
Lung	• Perform clinical screening for lymphangioleiomyomatosis (LAM) symptoms, including exertional dyspnea and shortness of breath, at each clinic visit. Counseling regarding smoking risk and estrogen use should be reviewed at each clinic visit for individuals at risk of LAM. • Obtain high-resolution computed tomography (HRCT) every 5-10 yr in asymptomatic individuals at risk of LAM if there is no evidence of lung cysts on their baseline HRCT. Individuals with lung cysts detected on HRCT should have annual pulmonary function testing (pulmonary function testing and 6-min walk) and HRCT interval reduced to every 2-3 yr. • mTOR inhibitors may be used to treat LAM patients with moderate to severe lung disease or rapid progression. TSC patients with LAM are candidates for lung transplantation but TSC comorbidities may impact transplant suitability.
Skin	• Perform a detailed clinical dermatologic inspection/exam annually. • Rapidly changing, disfiguring, or symptomatic TSC-associated skin lesions should be treated as appropriate for the lesion and clinical context, using approaches such as surgical excision, laser(s), or possibly topical mTOR inhibitor.
Teeth	• Perform a detailed clinical dental inspection/exam at minimum every 6 months and panoramic radiographs by age 7 yr, if not performed previously. • Symptomatic or deforming dental lesions, oral fibromas, and bony jaw lesions should be treated with surgical excision or curettage when present.
Heart	• Obtain an echocardiogram every 1-3 yr in asymptomatic pediatric patients until regression of cardiac rhabdomyomas is documented. More frequent or advanced diagnostic assessment may be required for symptomatic patients. • Obtain electrocardiogram (ECG) every 3-5 yr in asymptomatic patients of all ages to monitor for conduction defects. More frequent or advanced diagnostic assessment such as ambulatory and event monitoring may be required for symptomatic patients.
Eye	• Perform annual ophthalmologic evaluation in patients with previously identified ophthalmologic lesions or vision symptoms at the baseline evaluation. More frequent assessment, including those treated with vigabatrin, is of limited benefit and not recommended unless new clinical concerns arise.

TABLE 6: Presenting time of Clinical Manifestations [2, 32, 39-41]

	Clinical Feature	Time of onset
Present at birth	Hypopigmented macules	28 weeks gestation – 2 months
	Cortical tubers	18-30 weeks gestation
	SEN	18-30 weeks gestation
	SEGA	18 weeks gestation - Adolescence
	Cardiac Rhabdomyomas	22-26 weeks gestation
	Renal cysts	Infancy - adulthood
Late onset	Facial angiofibromas	2-5 years
	Fibrous cephalic plaques	2-5 years
	Shagreen patches	1st decade of life
	Confetti lesions	1st decade of life
	Renal angiomyolipomas	Childhood - Adulthood
	Retinal hamartomas	Birth - Adolescence
	Dental pits	Childhood - Adulthood
	LAM	3rd -4th decade of females

TABLE 7: Clinical Manifestations by age [41]

Presenting period of life	Clinical presentation
Fetal period	Seizures Cardiac arrhythmias
Neonatal period	Wolf-Parkinson-White syndrome – on ECG Hydrops fetalis Hypomelanotic macules Multiple renal cysts } On ultra sound Cardiac rhabdomyomas }
Infancy	Infantile spasms Retinal hamartomas Hypomelanotic macules Developmental delay
Early childhood	Autism Seizures Hypomelanotic macules
Late childhood	SEGA Facial angiofibroma Ungual fibroma
Adolescence	Shagreen patches
Adult	LAM, Renal angiomyolipoma

1.6. Phenotypic differences between *TSC1* and *TSC2*

Locus heterogeneity is a phenomenon where a disorder may due to mutations in more than one gene or locus [42]. When different mutations within the same gene at the same chromosome locus give rise to a single disease phenotype, it is defined as the allelic heterogeneity [42]. TSC has locus heterogeneity as *TSC1* at *9q34* and *TSC2* at *16p13.3* give

rise to the same disease. It is also an example for allelic heterogeneity as there are many gene mutations giving rise to the phenotype.

It is clear that there are overlapping clinical features among patients with *TSC1* mutations and *TSC2* mutations, but patients with *TSC1* mutations depicts a significantly less severe phenotype when compared to patients with *TSC2* mutations [19]. Some phenotypes such as grade 2-4 polycystic kidneys and angiomyolipomas, liver angiomyolipomas, retinal hamartomas and forehead plaques are virtually not seen in patients with mutations in the *TSC1* gene [19]. According to recent studies, patients with *TSC1* mutations have lower seizure frequencies, lower percentage of severe mental retardation, smaller number of subependymal nodules and cortical tubers and a lesser frequency of facial angiofibromas when compared to patients with *TSC2* mutations [8]. *TSC2* mutations are associated with early presentation of epilepsy, mainly infantile spasms [43], but recent studies report that missense mutations located in exon 23 to 33 of *TSC2* are associated with significantly less infantile spasms [29]. It is documented that the earlier the onset of seizures and the presence of infantile spasms, the poorer the prognosis. This group of patients are commonly reported with antiepileptic drug resistance, moderate to severe mental retardation and neuro cognitive phenotypes such as autism spectrum disorders which are significant in *TSC2* mutations [44].

Cystic kidneys can be present in patients with both *TSC1* and *TSC2* mutations. The more severe, early onset polycystic kidney disease results due to a contagious gene micro deletion syndrome resulting from a deletion in *TSC2* gene along with deletions in the adjoining *PKD1* gene [27]. It is also noted that symptomatic LAM is more frequently associated with *TSC2* mutations [32].

Among familial cases almost equal distribution between *TSC1* and *TSC2* can be seen while among sporadic cases *TSC2* mutations are about five times common than *TSC1* mutations [8]. *TSC1* mutations as well as the familial *TSC2* mutations are less severe in phenotype when

compared to *de novo TSC2* mutations. It is suggested this is due to more offspring in less severely affected patients with *TSC1* mutations [22]. Patients with no mutations in either gene had less severe phenotype than both *TSC1* and *TSC2* mutations [22]. In a study it was found that intellectual disability was more frequent in patients with *TSC2* mutations when compared to patients with *TSC1* mutations [45].

A broad spectrum of mutations has been identified in both genes varying from point mutations to large deletions, duplications and insertions. The mutations in *TSC1* are mostly point mutations such as nonsense mutations, small insertions and small deletions resulting in truncated proteins or inactivation of the protein synthesis. The mutation spectrum of *TSC2* comprises large deletions and rearrangements, small insertions and small deletions as well as point mutations [22].

1.7. Common mutation types in TSC

Nonsense mutation is a change in a single nucleotide that changes a single amino acid which eventually results a stop codon, resulting a truncated protein, usually an incomplete or a nonfunctional protein [42].

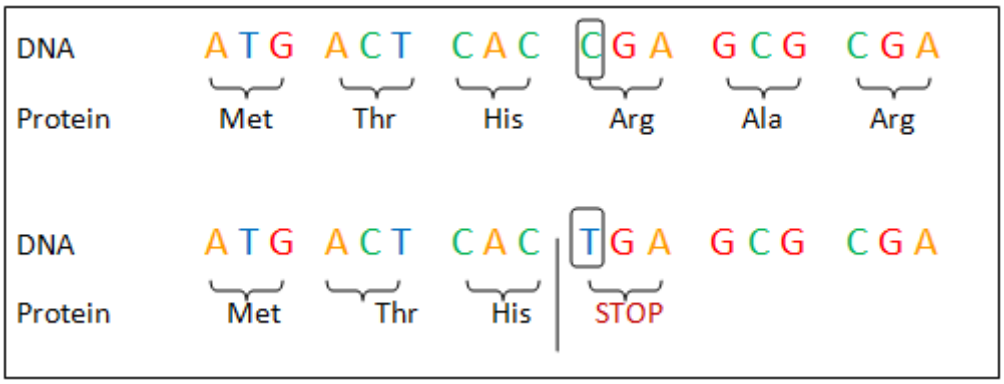


Fig 11: Replacement of cytosine by thymine has created a stop codon TGA result a truncated protein
C= Cytosine, T= Thymine, A= Adenine, G= Guanine

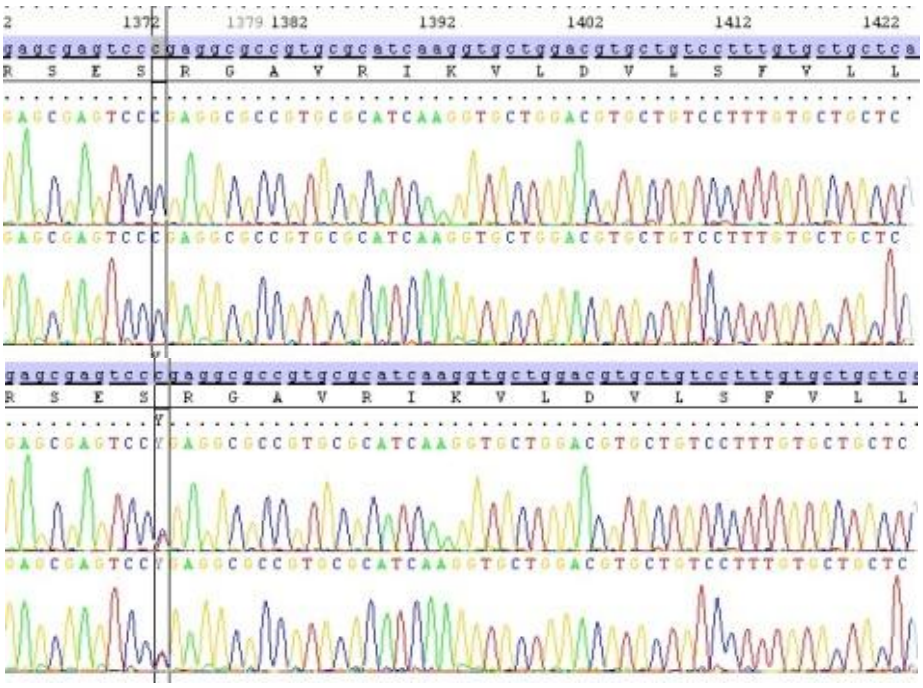


Fig 12: Sanger sequencing *TSC2* Exon 13 c.1372C>T nonsense mutation

Missense mutation is a change in a single nucleotide that changes only a single amino acid which changes the protein and the protein may or may not become nonfunctional due to the substitution of the different amino acid. Missense mutations account for more than half of all mutations [42].

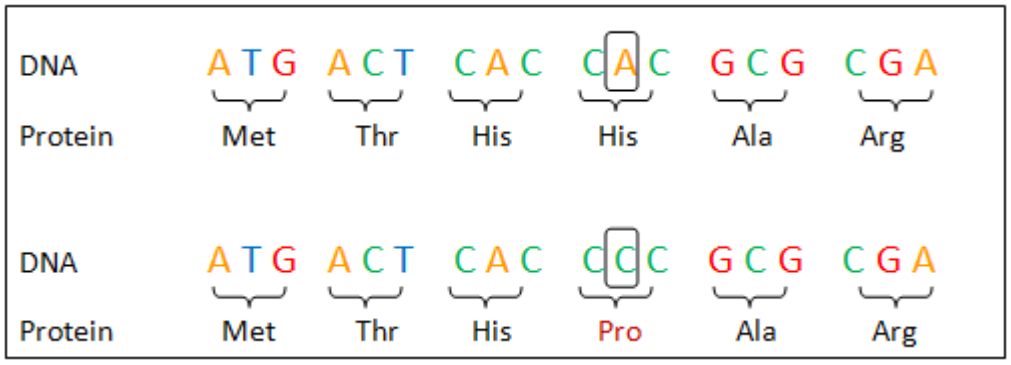


Fig 13: Replacement of adenine by cytosine changes the amino acid from Histidine to Proline, but does not result in a truncated protein

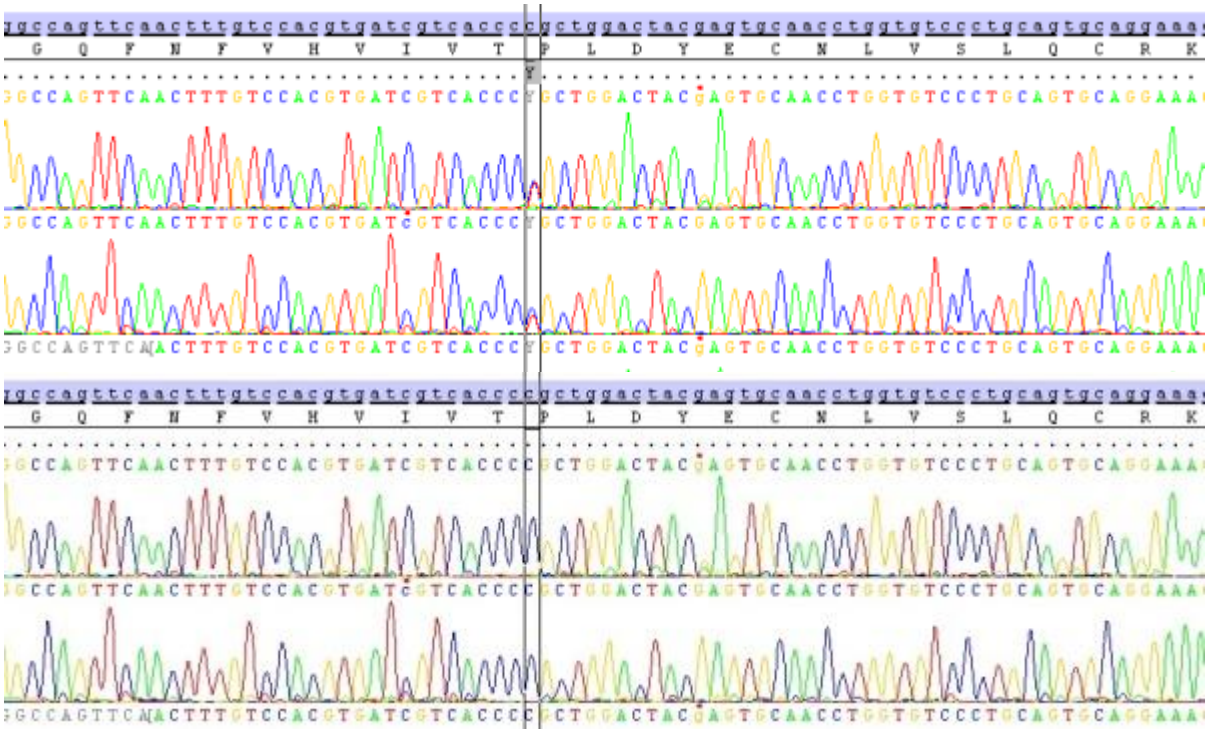


Fig 14: Sanger sequencing *TSC2* Exon 38 c.5024C>T missense mutation

Splice site mutation is a DNA change at a splicing site. During DNA transcription, intronic regions of DNA are spliced and only the exonic regions are present during the process of resulting mature messenger RNA (mRNA) from precursor mRNA. An insertion or a deletion of several nucleotides that disturbs a splice site may result in an incomplete splicing leading to aberrant protein product.

Fig 15: Sanger sequencing c.225 + 1 G>A splice site mutation

In-frame mutation is a mutation that does not change the triplet reading frame. Reading frame is a sequence of mRNA that translates into an amino acid chain, three bases (codon) at a time. A mutation that does not cause a shift in the triplet reading frame, but insertion/deletion of few amino acids either three or its multiples can change codons and there by the amino acid sequence resulting abnormal protein products.

Frame shift mutation changes the coding frame by insertion or deletion of several nucleotides that are not multiples of three, eventually disturbs the codons resulting in a completely different translation, thereby a completely different protein. Most frame shift mutations result in a premature stop codon downstream to mutation [42].

Fig 16: Sanger sequencing *TSC2* Exon 24 c.2764_2765 deletin of TT resulting a frame shift mutation.

Indels result from either insertion of few nucleotides or deletion of few. Insertion is an addition of one or more nucleotides. Deletion is a loss of DNA. Net result is a change in total number of nucleotides [42]. This results in deletion or insertion of amino acids or even a change in the reading frame leading to a different protein or a truncated protein. This protein may be non functional or with a change of function. Large deletions or insertions may result in unequal cross over during cell division leading to severe disease phenotype [42].

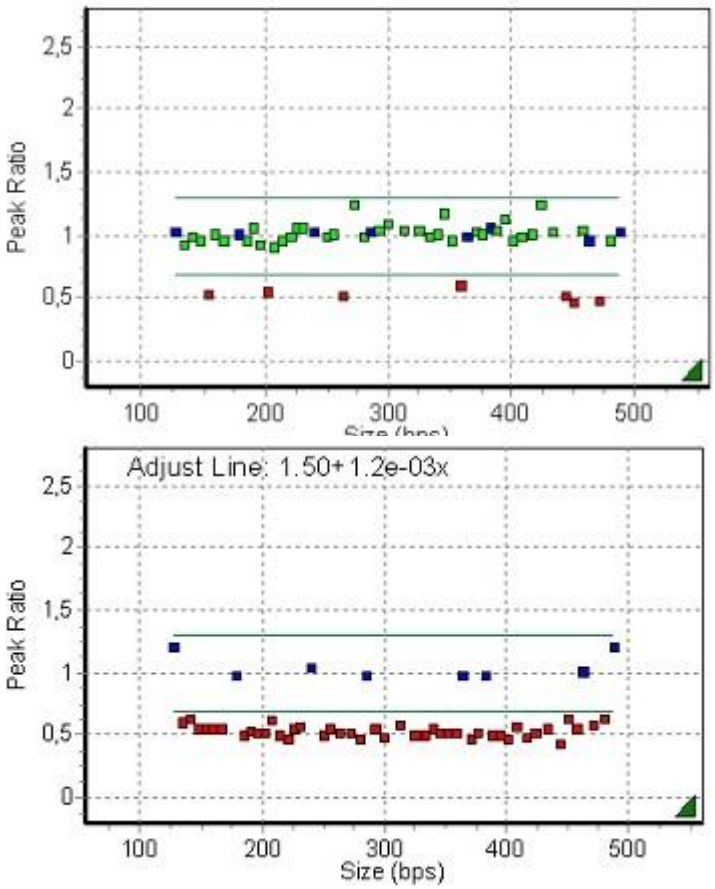


Fig 17: MLPA results, deletion of Exon 33 to 36 (above) and haplo insufficiency of *TSC2* (below)

Frequencies of different types of mutations found in *TSC1* and *TSC2* were documented in a study done in 2005 [22]. Figure 18 shows the results of this study.

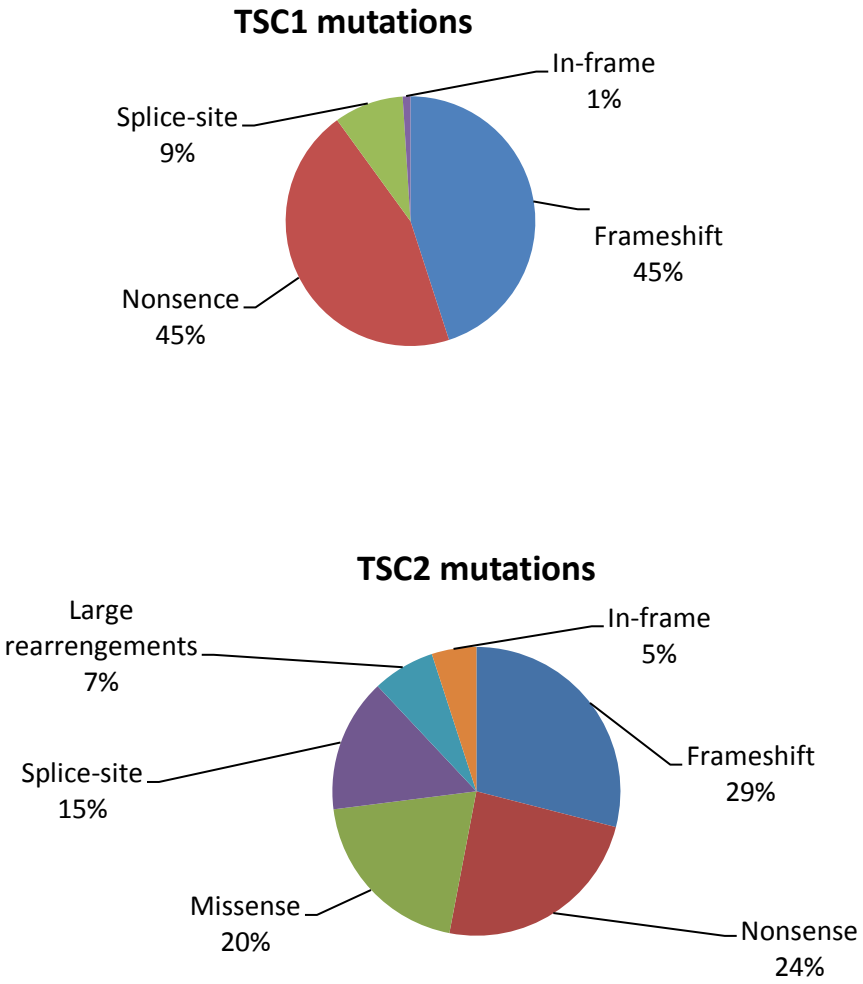


Fig 18: Different types of mutations in *TSC1* and *TSC2* genes

2. Justification of a study on Tuberous Sclerosis Complex in a Sri Lankan cohort

2.1. Importance of this study

Tuberous Sclerosis Complex is no more considered a rare disorder. Recent studies estimate an incidence rate between 1 in 6,000 to 1 in 10,000 live births with a population prevalence of 1 in 20,000 [2].

A study done in 2009 and few case reports have confirmed the presence of TSC in Sri Lanka [46-48].However a study regarding the mutations in TSC genes in Sri Lankan patients has not been conducted. Therefore genotype- phenotype correlation among the Sri Lankan patients is not yet known.

From the view of the health care system, phenotyping the TSC patients in Sri Lanka is necessary for healthcare planning with a multidisciplinary approach. Phenotyping TSC is a practically challenging process because of its numerous and diverse clinical signs, its variable expressivity and the age related nature of some clinical manifestations. Phenotyping patients with TSC in Sri Lanka will provide them with information on their disease and prognosis. This process will also contribute to the development of a management protocol which is both according to the international guidelines and also suitable for the Sri Lankan health care system.

Genotyping *TSC1* and *TSC2* is also a challenging process as there are said to be more than 1000 disease causing mutations and the number of new mutations are constantly increasing. Although there has been a steady increment in understanding the phenotypic and genotypic variation associated with TSC during the last two decades, there is paucity with regard to the genotype of the Sri Lankan patients. Obtaining three generation family history is beneficial in

diagnosing family members with milder phenotype. Genetic testing is also beneficial for the patients as they can get a better knowledge with regard to the disease with pre-test and post-test counselling apart from the genetic diagnosis. We will be able to counsel them on diagnosis, prognosis, reproductive options and future management plans. Awareness of the parents will probably lead to better care for the affected children; especially in those with epilepsy. Patients with a genetic diagnosis will also be able to participate in research and clinical trials leading to new treatment. Academically, genotyping is beneficial as we can correlate the phenotype with the genotype and compare it with the rest of the world. We will also be able to look for founder mutations specific to Sri Lankan patients and novel mutations that are clinically significant. This will be beneficial for the general knowledge of TSC.

Further development of this project can lead to the establishment of an advocacy group such as Tuberous Sclerosis Alliance group in USA, so that both the patients and clinicians can benefit from management of the TSC patients in Sri Lanka as well as rest of the world.

2.2. Objectives

The main objective of the work presented in this thesis is to clinically and genetically characterize TSC patients in Sri Lanka. The second objective is to correlate the genotype with phenotype and to establish a genotype-phenotype spectrum in Sri Lankan patients.

Objectives:

1. To describe the clinical phenotype of TSC in a cohort of Sri Lankan children
2. To study the mutations in *TSC1* and *TSC2* in Sri Lankan children with TSC
3. To identify the pattern of mutations in *TSC1* and *TSC2* giving rise to infantile spasms and other seizure types

4. To identify *PKD1* gene deletions in TSC patients with polycystic kidney disease and to compare with the known deletions
5. To compare Sri Lankan genotypes with genotypes from patients with TSC from other countries

Ethical considerations

The ethical review committee of Faculty of Medicine, University of Colombo, Sri Lanka has approved the study.

The study was conducted according to the Declaration of Helsinki (2008) and is a collaborative effort between the Human Genetics Unit, Faculty of Medicine, Colombo, patients with TSC, paediatric neurologists, paediatric cardiologists, dermatologists and paediatricians at the tertiary care hospitals in Sri Lanka and the Department of Medical Genetics, University of Oslo, Norway, Norwegian Epilepsy Center, Sandvika and the Norwegian center for rare epilepsy-related disorders, Oslo University Hospital. The study has social value as this is the first effort to correlate phenotype with genotype in Sri Lankan children with TSC and therefore contributes to the general knowledge in the field. The study was designed to ensure scientific validity. The study was opened to all patients with a clinical diagnosis of TSC who falls into the age group of 0 – 18 years.

Appropriate measures were taken to ensure that consent is obtained in an ethical manner from all study participants. Written informed consent was obtained from parents or guardians (proxy consent) using information sheets (Appendix 3) and consent forms (Appendix 3) in Sinhala, Tamil and English languages according to their preference. The patients and their parents/guardian were interviewed privately to ensure privacy and were able to discuss the study privately with the principal investigator without the presence of others.

Parents/guardians were able to make a decision to participate their children to the study without external interference.

The data collection booklet (Appendix 3) was designed to ensure confidentiality of information gathered. Soon after collecting the personal information, the identification page was removed and filled separately. The only identification number in the rest of the booklet is a coded subject study number which cannot be linked to an individual without the page containing the personal information which was kept by the principal investigator under lock and key. The electronic database containing the clinical data only had the subject study number, thus ensuring confidentiality. The database and the computer containing the database were password protected.

Photographs of some clinical manifestations were captured by the principal investigator only after proper proxy consent was obtained. Full anonymity was maintained by covering the eyes where the face is exhibited. Name of the patient was not mentioned in images such as the 2D echocardiograms. The small risk of complications, such as bruising or infection after the venaepuncture was minimized as it was performed under aseptic conditions by the principal investigator or a trained nurse.

Benefits to the patient included improved knowledge on the disease and also in the cases where we could establish a genetic diagnosis, they receive a molecular diagnosis. All the parents of the participants will be counseled and advised on family planning, pre-conception genetic testing and life style modification of their children with TSC according to the test results.

The samples and data obtained will be stored for further studies in TSC until 2024. Thereafter the remaining samples will be anonymised and discarded by the laboratory under the supervision of the investigator. Appropriate consent has been obtained for this purpose and such studies would be subjected to ethics review prior to conduct.

3. Material and methods

This study was conducted in a paediatric population age ranging from 0 – 18 years. All the patients presented to paediatric neurology units, paediatric cardiology units at LRH and other teaching hospitals were recruited into the study. Patients were recruited from October 2013 to March 2014.

3.1. Study population

Inclusion and exclusion criteria for the study were as below:

Inclusion criteria

1. Clinical diagnosis of TSC; two major criteria or one major with two or more minor criteria according to Table 2
2. Ability to provide proxy consent from parent/guardian
3. Ability to obtain a valid history of epilepsy and/or polycystic kidney disease with TSC
4. Sri Lankan origin

Exclusion criteria

Subjects with TSC with other reasons for epilepsy such as head injury, epileptic encephalopathy etc.

Patients' parents were contacted via phone/mail and those who gave informed proxy consent were recruited into the study. Data for a total of 44 patients were included in to the study and recorded in the data base.

Recruitment of Patients

Identification and recruitment of the patients with Tuberous Sclerosis Complex was conducted in the following manner;

1. Identification from the database at the professorial Paediatric Neurology Unit at Lady Ridgeway Hospital for Children (LRH), Colombo.

2. Sending a call letter to all the paediatric neurologists, paediatric cardiologists, dermatologists and paediatricians at the tertiary care centers around the country requesting to refer patients with TSC into the study.

3. Retrieving the archives of Human Genetics Unit, Faculty of Medicine, Colombo from 2005 to 2013 for patients with TSC.

4. Paying home visits for the children who are not visiting the hospitals, but being registered at neurology, cardiology or dermatology clinics at tertiary care centers.

3.2. Registration of the Patients

A data base was established including gender, presenting features, three generation pedigree, clinical manifestations, investigation findings (MRI, CT, EEG, ultra sound scans, genetic diagnosis), social status and treatment regimes. Each index case was registered in this data base. The 44 patients were from 42 families as two patients were twins and two were siblings.

3.3. Clinical Evaluation

Detailed medical history was obtained from parents/guardian of each patient. To minimize recall bias, the history was obtained from two adults closely in contact with the patient whenever possible. Family history was obtained both from the mother and the father whenever possible. A thorough medical examination was done with emphasis on diagnostic

criteria of TSC by the principal investigator and all previous and relevant investigations were evaluated. The findings were reassessed by the supervisors.

Diagnosis of epilepsy was classified according to International League Against Epilepsy (ILAE) classification 2010 [49]. Infantile spasms were confirmed by referring the clinical notes, EEG findings and detailed history from parents/guardian. According to the ILAE proposals, failure of adequate trials of two or more tolerated and appropriately chosen anti epileptic drug schedules is considered as patients with intractable epilepsy [50].

ILAE classification 2010

Classification is essential to understand, diagnose and manage epilepsy. Depending on the etiology the treatment regime and management plan differs. I.e. Vigabatrin is the drug of choice in infantile spasms in patients with TSC.

The International League Against Epilepsy (ILAE) was established in 1909 with a mission of supporting health professionals, patients and their care givers, governments and public with all aspects related to epilepsy.

ILAE has revised the classification and terminology for organization of seizures and forms of epilepsy in 2010 (Appendix 2). This new classification has revised the concepts, terminology and approaches of the previous classifications published in 1981 on epileptic seizures and in 1989 on epileptic syndromes [51].

With new classification all seizures fall into generalized, focal or unknown type. The terminology and concepts have also changed towards highlighting genetic aetiology instead of idiopathic epilepsy. TSC falls into structural-metabolic aetiology of seizures. Infantile spasms are classified as brief contractions lasting 70-200ms involving any group of muscles. Other seizures in TSC are usually focal in origin. The ILAE classification is freely available on line at http://www.ilae.org.

Electroencephalogram

Electroencephalogram (EEG) is one of the main diagnostic tests used in epilepsy and is also useful in diagnosis of some brain disorders such as tumors, stroke, coma, encephalopathy and brain death [52]. EEG records the changes in electrical activity in different regions of the brain with the help of small probes attached to the scalp [53].

In this study, the EEG report prior to treatment and the subsequent EEG reports were taken in consideration when classifying seizures in the patients. The EEG pattern in patients with TSC and IS not necessarily similar to the EEG pattern in patients with West syndrome (IS, hypsarrhythmia in EEG and developmental delay) [29, 54]. Therefore, apart from the EEG reports, the clinical notes describing the seizure semiology and the history from the parents and or guardians werealso considered in determining the seizure types.

Neuro imaging

Magnetic resonance imaging (MRI) is the standard recommended imaging technique to diagnose cortical tubers and other cortical dysplasias, SEN and SEGAs according to international guidelines [1]. In this study population MRI scanning was available only in 17 patients. In 25 patients, computerized tomography (CT) was available and 2 patients who were below 6 months of age had ultrasound scan brain and were awaiting CT examination of the brain.

Ultra sound scan of the abdomen

All patients were screened with ultrasound scan (USS) abdomen to look for angiomyolipomas in abdominal viscera and renal cysts at the time of diagnosis. Subsequent scans were done according to the international guide lines depending on the state of the first scan [1]. In this study, we reviewed the report from the most recent scan confirm or exclude the relevant diagnostic criteria.

Two Dimensional Echocardiogram

At least one Two Dimensional Echocardiogram (2D Echo) was available in all 44 patients done at the time of diagnosis. The first 2D Echo findings were considered in this study in order to identify the earliest possible findings as cardiac rhabdomyomas may be congenital in TSC.

3.4. Genetic Testing

DNA extraction

At recruitment, 5ml of venous blood was collected in K/EDTA tubes from each patient. The samples were stored at -80°C at the Human Genetics Unit, until the DNA extraction was carried out. DNA was extracted using the QIAamp DNA Blood Mini Kit (250) by QIAGEN® (Venlo, Netherlands). Extracted DNA was stored at -20°C until it was shipped to Oslo, Norway. DNA was quantified using NanoDrop 8000 UV-Vis Spectrophotometer (Thermo Scientific).

Polymerase Chain Reaction

Before sequencing the genes, it is necessary to amplify the number of copies of the target sequence with a Polymerase Chain Reaction (PCR). Using this biochemical technology, single or limited numbers of copies of DNA are amplified in to many folds resulting in thousands to millions of copies of the DNA sequence that we are interested in.

The method is based on two primers annealing to complementary sequences at either end of the target sequence. DNA polymerase synthesizes DNA, elongates the primers and makes two new duplicates of the original template. In order to do this, PCR is dependent on a DNA template, specific primers, DNA polymerase enzyme, deoxynucleotide triphosphates (dNTPs) and thermal cycling [55].

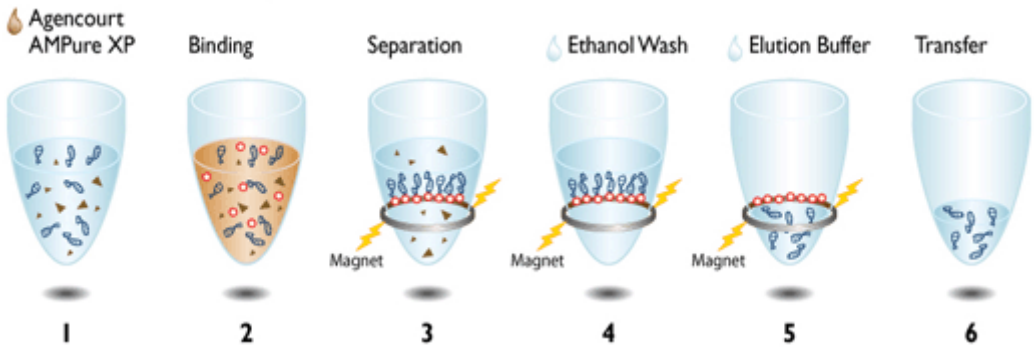


Fig19: PCR reaction 2. Binding of PCR amplicons to magnetic beads 3. Separation of PCR amplicons bound to magnetic beads from contaminants 4. Washing of PCR amplicons with Ethanol 5. Elution of PCR amplicons from the magnetic particles 6. Transfer away from the beads into a new plate (https://www.beckmancoulter.com)

Primer design

The reference sequence used for *TSC1* and *TSC2* were NM_000368.4 and NM_000548.3 respectively, and were downloaded from Ensembl (http://www.ensembl.org/). Primers are short oligonucleotides that are complementary to the end of a target sequence, and have to be unique for each target sequence. The primer design for *TSC1* and *TSC2* was carried out at the Department of Medical Genetics at the University Hospital in Oslo using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Full primer sequence is attached in appendix 4.

Sanger sequencing

Once the target DNA was amplified, the sequencing process can start. Sanger sequencing is a DNA sequencing method based on selective incorporation of chain terminating dideoxynucleotides (ddNTDs) by DNA polymerase during in vitro DNA replication [56]. The technology is used to determine the order of the nucleotides in a DNA sequence, and also, as in this project, detect single nucleotide variation (SNV) and small deletions or insertions.

Then the sequencing reaction is run on an automated Applied Biosystems 3730xl DNA Analyzer for electrophoresis and data analysis. During electrophoresis, DNA molecules in the reaction are injected into a capillary array. The injected DNA molecules are then separated

through the array according to size. Each fragment is detected by the fluorescent tag, present on each ddNTD that were incorporated during the sequencing reaction. It appears as a colored peak on data analysis software. Different ddNTDs give four different colors depending on the attached nucleotide. The data analysis software interprets raw data peaks and automatically generates the final sequence of DNA template.

Multiplex Ligation dependent Probe Amplification

Multiplex Ligation dependent Probe amplification (MLPA®) is a high-throughput method to determine copy number variations (CNVs) in genomic sequences [57]. With MLPA we can detect larger deletions and rearrangements than with Sanger sequencing, i.e. these two methods fulfill each other when searching for a genetic cause for disease.

In the MLPA method it is not the sample DNA that is amplified during PCR reaction, but MLPA probes that hybridize to the target DNA sequence [58]. Each MLPA probe consists of two oligonucleotide probes that hybridize adjacently to target. Only the probes that have ligated are amplified by PCR. One pair of primers is used per MLPA PCR reaction. The relative number of fragments present after PCR reaction depends on the relative amount of the target sequence present in a DNA sample. MLPA® comes as a commercial kit solely produced by MRC – Holland (Amsterdam, the Netherlands) [59]. Samples that showed negative results and the samples that are inconclusive after Sanger sequencing were analyzed by MLPA.

3.5 Analysis of Results

Mutation analysis done by referring Leiden Open Variation database(LOVD) (www.lovd.nl/TSC1, www.lovd/TSC2), Human Gene Mutation (HGMD) database (http://www.hgmd.org/) and ClinVar database(http://www.ncbi.nlm.nih.gov/clinvar/). Gene protein effects were analyzed using Mutalizer 2.0.beta – 31 database (https://mutalyzer.nl). For the mutations that were not reported in these databases, a literature search was done using PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and Google scholar (http://scholar.google.no/).

4. Results

The total number of patients in the study population was 44 from 42 families. Among them 34 (77.3%) were Sinhalese, 6 (13.6%) were Sri Lankan Tamil and 4 (9.1%) were Moor. There were 24 (54%) males and 20 (46%) females, aged 9 days to 18 years with a mean age of 6.5 years and a median age of 4.5years (standerd deviation +/- 2.4). Among the patients at least one clinical feature was present at birth in 31 (70.4%) patients while late presentations were noted in 13 (29.6%) patients.

The most common clinical feature present at birth was hypopigmented macules, which was the presenting clinical sign in 26 (84%) patients. Three patients (10%) presented with cardiac murmurs or arrhythmia and one patient (3%) presented with gross ascites on day one of life due to renal failure. All these 4 patients also had hypopigmented macules in addition to their main presenting complaint. One patient (3%) presented with a cephalic plaque at birth.

Neurobehavioral phenotypes were not taken into account as psychiatric assessment was not being done as a routine part of evaluation of patients with TSC in Sri Lanka.

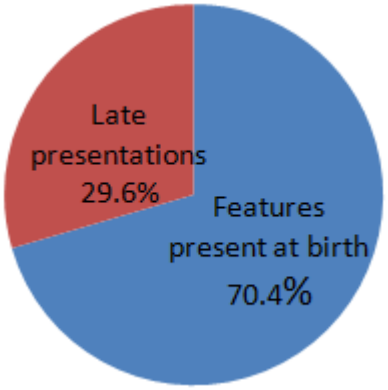


Fig 20: Presentation of clinical features in study population

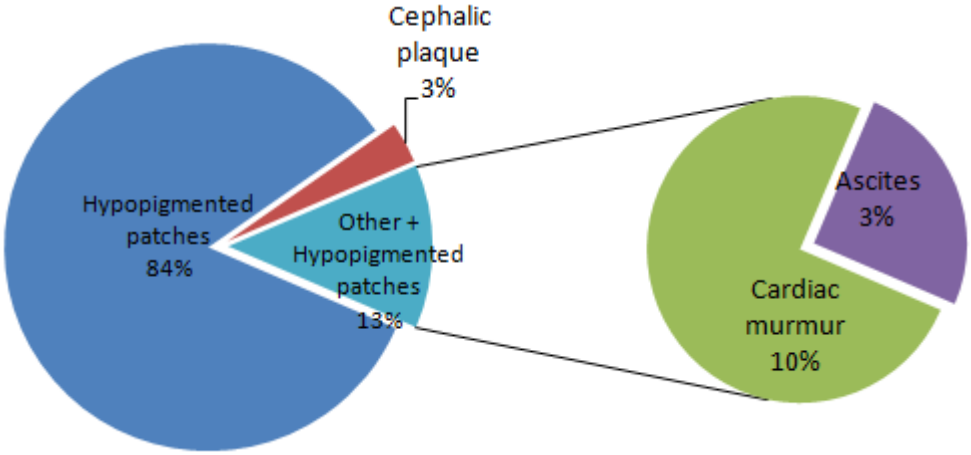


Fig 21: Clinical features present at birth in study population

4.1 Phenotype of the sample population

Table 8, 9 and 10 sumarises the phenotype of 44 patients in the study population.

TABLE 8: Skin manifestations of the study population

Pt No	FH	Skin manifestations					
	FH of TSC	Hypopig:macules	Angiofibroma	Fibrous Ce:plaques	Ungual fibroma	Shagreen patch	Confetti lesions
01-1	N	Y	N	N	N	Y	N
02-1	Y	Y	N	N	N	Y	N
03-1	Y	Y	Y	N	N	Y	N
04-1	Y	Y	Y	N	N	N	N
05-1	Y	Y	Y	Y	Y	N	N
06-1	Y	Y	Y	Y	N	N	N
07-1	Y	Y	N	N	N	Y	N
08-1	Y	Y	Y	N	N	N	N
09-1	Y	Y	Y	Y	N	Y	N
10-1	N	Y	N	N	N	N	N
11-1	N	Y	N	N	N	N	N
12-1	N	Y	N	N	N	N	N
13-1	N	Y	N	N	N	Y	N
14-1	Y	Y	N	N	N	N	N
15-1	Y	Y	N	N	N	N	N
16-1	N	Y	Y	Y	N	N	N
17-1	N	Y	Y	Y	N	Y	N
18-1	N	Y	N	N	N	N	N
19-1	N	Y	N	N	N	N	N
20-1	N	Y	Y	Y	N	N	N
21-1	N	Y	Y	Y	N	N	N
22-1	N	N	N	Y	N	N	N
23-1	N	N	Y	N	N	N	N
24-1	N	Y	N	N	N	N	N
25-1	N	Y	Y	N	N	N	N
26-1	N	Y	Y	Y	N	Y	N
27-1	N	Y	Y	N	N	N	N
28-1	N	Y	Y	Y	N	Y	N
29-1	N	Y	N	N	N	N	N
30-1	N	Y	N	Y	N	Y	N
31-1	Y	Y	N	N	N	N	N
32-1	Y	Y	N	N	N	N	N
33-1	Y	Y	Y	N	N	N	N
34-1	N	Y	N	N	N	N	N
35-1	N	N	Y	Y	N	N	N

Pt No	FH	Skin manifestations					
	FH of TSC	Hypopig:macules	Angiofibroma	Fibrous Ce:plaques	Ungual fibroma	Shagreen patch	Confetti lesions
36-1	N	Y	N	N	N	Y	N
37-1	Y	N	Y	Y	N	Y	N
38-1	Y	N	Y	N	Y	N	N
39-1	N	Y	N	Y	N	N	N
40-1	Y	Y	Y	Y	N	N	N
41-1	N	Y	Y	N	N	N	N
42-1	Y	Y	N	N	N	Y	N
43-1	N	Y	N	N	N	N	N
44-1	N	Y	N	N	N	N	N
Total	**17**	**39**	**21**	**15**	**2**	**13**	**0**
%	**38.6**	**88.6**	**47.7**	**34**	**4.5**	**29.5**	**0**

Pt No = Patient number, FH = Family History, Hypopig:macules = hypopigmented macules, Fibrous ce:plaques = fibrous cephalic plaques, Y = present, N = absent, % = percentage

TABLE 9: Brain manifestations of the study population

Pt No	Brain manifestations							
	Seizures					Radiological findings		
	Infantile spasm	Generalized	Focal	Intractable	FH	Tubers	SEN	SEGA
01-1	Y	N	Y	Y	N	Y	N	N
02-1	Y	N	Y	Y	N	Y	N	N
03-1	Y	N	Y	Y	Y	Y	Y	N
04-1	Y	N	Y	N	Y	Y	Y	N
05-1	Y	Y	N	N	Y	Y	Y	N
06-1	Y	Y	Y	Y	Y	Y	Y	Y
07-1	Y	N	Y	Y	Y	Y	Y	N
08-1	Y	N	Y	N	Y	Y	N	N
09-1	N	N	Y	N	Y	N	Y	N
10-1	Y	N	Y	Y	N	-	-	-
11-1	N	Y	N	N	Y	-	Y	-
12-1	Y	N	Y	Y	N	Y	Y	N
13-1	N	N	Y	N	N	Y	Y	N
14-1	N	N	N	N	Y	-	-	-
15-1	N	N	Y	N	Y	N	Y	N
16-1	N	N	Y	N	N	-	-	-
17-1	Y	N	Y	N	N	Y	Y	N
18-1	N	Y	N	N	N	Y	Y	N
19-1	Y	N	Y	N	N	-	-	-
20-1	Y	N	Y	N	N	Y	Y	N
21-1	Y	N	Y	Y	N	Y	N	N
22-1	Y	Y	N	N	N	Y	Y	N
23-1	N	Y	N	Y	N	Y	Y	N
24-1	Y	N	Y	N	N	N	Y	N
25-1	N	N	Y	N	N	Y	Y	N
26-1	N	N	Y	Y	N	Y	Y	N
27-1	N	N	Y	N	N	Y	Y	N
28-1	N	N	Y	Y	N	Y	Y	N
29-1	N	N	Y	N	N	Y	Y	N
30-1	N	N	N	N	N	-	-	-
31-1	N	N	Y	N	N	Y	Y	N
32-1	Y	N	Y	N	Y	Y	N	N
33-1	N	N	Y	N	Y	N	N	Y
34-1	N	N	Y	Y	N	Y	Y	N
35-1	N	N	Y	N	N	N	Y	N

Pt No	Brain manifestations							
	Seizures					Radiological findings		
	Infantile spasm	Generalized	Focal	Intractable	FH	Tubers	SEN	SEGA
36-1	N	Y	Y	Y	N	Y	Y	N
37-1	N	N	Y	N	Y	Y	Y	N
38-1	N	N	Y	Y	N	N	Y	N
39-1	N	N	Y	Y	N	N	N	N
40-1	N	N	Y	Y	N	Y	Y	N
41-1	Y	N	Y	Y	N	Y	Y	N
42-1	N	N	N	N	N	-	-	-
43-1	N	N	Y	N	N	-	-	-
44-1	Y	N	Y	N	N	N	Y	N
Total	**19**	**7**	**36**	**17**	**13**	**28**	**30**	**2**
%	**43.1**	**15.9**	**81.8**	**38.6**	**29.5**	**63.6**	**68.1**	**4.5**

Pt No = Patient number, FH = Family History of seizures, Y = present, N = absent,
% = percentage

TABLE 10: Other manifestations of the study population

Pt No	Other Manifestations								
	Kidney		Liver	Eye		Heart	Lung	Mouth / Teeth	
	Angio-myolipoma	Cysts	Angio-myolipoma	Retinal hamartoma	Acromic patch	Rhabdo-myoma	LAM	Enamel pit	Intra oral fibroma
01-1	G2	N	N	N	N	N	N	N	N
02-1	N	N	N	N	N	N	N	N	N
03-1	N	N	N	N	N	Y	N	Y	N
04-1	N	N	N	N	N	N	N	N	N
05-1	N	G3	N	N	N	N	N	N	N
06-1	N	G2	N	N	N	Y	N	N	N
07-1	N	N	N	N	N	Y	N	Y	N
08-1	N	N	N	Y	N	N	N	N	N
09-1	N	N	N	N	N	Y	N	N	N
10-1	N	N	N	N	N	Y	N	-	N
11-1	N	N	N	Y	N	-	N	-	-
12-1	N	N	N	N	N	N	N	N	N
13-1	N	N	N	N	N	Y	N	N	N
14-1	N	N	N	-	-	Y	N	-	-
15-1	N	G3	N	N	N	Y	N	Y	N
16-1	N	N	N	N	N	N	N	N	N
17-1	N	N	N	N	N	N	N	N	N
18-1	G3	N	N	N	N	Y	N	N	N
19-1	-	-	-	N	N	N	N	N	Y
20-1	N	N	N	-	-	-	-	N	N
21-1	N	N	N	N	N	N	N	N	N
22-1	-	-	-	-	-	-	-	Y	N
23-1	N	N	N	N	N	N	N	N	N
24-1	G1	N	N	N	N	N	N	N	N
25-1	N	G4	N	N	N	N	N	Y	N
26-1	N	N	N	N	N	N	N	N	N
27-1	N	N	N	N	N	N	N	N	N
28-1	N	N	N	Y	N	N	N	Y	N
29-1	-	G4	-	-	-	Y	N	N	N
30-1	N	N	N	N	N	N	N	N	N
31-1	G2	N	N	N	N	N	N	N	N
32-1	N	N	N	N	N	N	N	N	N
33-1	N	N	N	N	N	Y	N	N	N

	Other manifestations								
Pt No	Kidney		Liver	Eye		Heart	Lung	Mouth / Teeth	
	Angio-myolipoma	Cysts	Angio-myolipoma	Retinal hamartoma	Acromic patch	Rhabdo-myoma	LAM	Enamel pit	Intra oral fibroma
34-1	G1	N	N	N	N	Y	N	N	N
35-1	N	N	N	-	-	-	-	N	N
36-1	N	G2	N	-	-	N	N	N	N
37-1	N	N	N	N	N	N	N	N	N
38-1	N	N	N	N	N	Y	N	N	N
39-1	N	N	N	-	-	N	N	N	N
40-1	N	G3	N	-	-	N	N	N	N
41-1	N	N	N	-	-	Y	N	N	N
42-1	N	N	N	-	-	Y	N	N	N
43-1	-	-	-	-	-	Y	N	N	N
44-1	N	N	N	N	N	N	N	N	N
Total	**5**	**6**	**0**	**3**	**0**	**16**	**0**	**6**	**1**
%	**11.3**	**13.6**	**0**	**6.8**	**0**	**36.3**	**0**	**13.6**	**2.2**

Pt No = Patient number, Y = present, N = absent, %= Percentage, G1, G2 , G3& G4 = Grading of AML and RC according to Dabora et al [19].(Appendix 2)

4.2 Genotype of the study population

As two pairs of patients were twins and siblings, the mutations of one pair are considered as one. Therefore the total number of mutations considered was 42. With Sanger sequencing 20 mutations were detected in *TSC2* and with MLPA another 7 deletions were detected adding the total number to 27 mutations (64.2%). In *TSC1*, 8 mutations were detected with Sanger sequencing, while one mutation was detected by MLPA. The total mutations found in *TSC1* were 9 (21.5%). In the whole sample population mutation detection rate was 85.7%. No mutation was detected in 6 patients (14.3%).

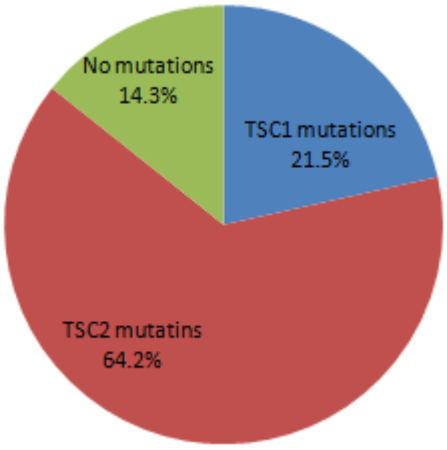


Fig 22: mutation pattern in Sri Lankan study population

Table 11 shows the genotype of the study population.

TABLE 11: Genotype of TSC patients in this study

	Location	Mutation	Effect	Predicted effect	Inheritance by Clinical Evaluation	Reports
1	TSC2 Ex 33-35	DEL EX33-35	deletion	Protein truncation	Sporadic	novel
2	TSC2 Ex 38	c.5024C>T	missense	p.Pro1675Leu	Familial	[60]
3	TSC2 Ex 13	c.1372C>T	nonsense	p.Arg458*	Familial	[61]
4	TSC2 Ex 13	c.1372C>T	nonsense	p.Arg458*	Familial	[61]
5	TSC2 Ex 38	c.5024C>T	missense	p.Pro1675Leu	Familial	[60]
6	TSC2 Ex 38	c.5024C>T	missense	p.Pro1675Leu	Familial	[60]
7	TSC2 Ex 33	c.4442dupA	duplication	p.Ser1482Glufs*42	Familial	novel
8	TSC2 Ex 38	c.5024C>T	missense	p.Pro1675Leu	Familial	[60]
9	TSC1 Ex 17	c.2074C>T	nonsense	p.Arg692*	? Familial	[62]
10	TSC2 Ex 24	c.2764_2765del	deletion	p.Leu922Valfs*3	Sporadic	novel
11	TSC2 Ex 40	c.5237A>C	missense	p.His1746Pro	Sporadic	novel
12	TSC1 Ex 18	c.2356C>T	nonsense	p.Arg786*	*De novo*	novel
13	TSC1 Ex 18	c.2356C>T	nonsense	p.Arg786*	*De novo*	novel
14	TSC1 Ex 17	c.2074C>T	nonsense	p.Arg692*	? Familial	[62]
15	TSC2 Ex 39	c.5114A>G	nonsense	p.Asp1705Gly	*De novo*	novel
16	No variants					
17	TSC2 Ex 40	c.5227C>T	nonsense	p.Arg1743Trp	Sporadic	[63]
18	TSC2 Ex 14	c.1528C>T	nonsense	p.Gln510*	Sporadic	novel
19	TSC2 Ex 16	c.1832G>A	missense	p.Arg611Gln	Sporadic	[37]
20	TSC2 Ex1,UPS	DEL UPS-EX1	deletion	?protein truncation	Sporadic	novel
21	TSC2 Ex 3	c.270del G	deletion	p.Gln90Hisfs*16	Sporadic	novel
22	No variants					
23	TSC2 Ex 27	c.3280C>T	missense	p.Ser1094Leu	Sporadic	novel
24	TSC2 Ex 23	c.2713C>T	missense	p.Arg905Trp	Sporadic	[64]
25	Multigene	DEL TSC2 + PKD1	con.gene del	haplo insuficiency	Sporadic	[65]
26	TSC2 Ex 15	c.1690_1693delGTCC	deletion	p.Val564Trpfs*133	Sporadic	novel
27	TSC2 Ex 15	DEL EX15	deletion	Protein truncation	Sporadic	novel
28	TSC2 Ex 17	C.1864C>T	missense	p.Arg622Trp	Sporadic	[66]
29	Multigene	DEL TSC2 + PKD1	con.gene del	haplo insuficiency	Sporadic	[65]
30	No variants					
31	No variants					
	Location	Mutation	Effect	Predicted effect	Inheritance by Clinical Evaluation	Reports
32	TSC1 Ex 5	c.362A>G	missense	p.Lys121Arg	Familial	novel
33	TSC1 Ex 10	c.989dupT	frame shift	p.Ser331Glufs*10	Familial	novel
34	TSC2 Int 2A	c.225+1G>A	spl.site del	splicing efect	Sporadic	novel

35	No variants					
36	TSC1 Ex 9	DEL EX9-23	deletion	Protein truncation	Sporadic	novel
37	TSC2 Ex 3	DEL EX3A-14	deletion	Protein truncation	Familial	novel
38	TSC2 Ex 23	c.2688delG	frame shift	p.Trp896Cysfs*52	Familial	novel
39	No variants					
40	TSC2 Ex 36	DEL EX36-41 +PKD1	deletion	Protein truncation	Familial	novel
41	TSC2 Ex 20	c.2251C>T	nonsense	p.Arg751*	Sporadic	[62]
42	TSC1 Ex 15	c.1498C>T	nonsense	p.Arg500*	? Familial	[67]
43	TSC2 Ex 29	c.3571_3581del	frame shift	p.Thr1191Glyfs*39	Sporadic	novel
44	TSC2 Ex 35	c. 4589C>A	frame shift	p.Ser1530*	Sporadic	novel

Highlighted in yellow – novel mutations, con.gene del = contagious gene deletion, ?Familial = presence of clinically affected family members, but presence of reported data with *De novo* cases, *De novo* = parents tested, no mutations found

There were some normal variants previously reported in studies as well as in databases. In this study, in *TSC1*, they were c.965T>C [68], c.1335A>G [62] and c.1460C>G [66]. In *TSC2*, they were c.1100G>A, c.1578C>T, c.2580T>C, c.5017G>C [68], c.4536C>T and c.5161-10A>C [62].

There were some variants which are apparently neutral as they were present in more than 6 patients apart from well documented pathogenic mutations, though there is no reported data strong enough to conclude they are normal variants. In *TSC1*, they were c.1218C>T, c.1333+13C>T and c.3387C>T. In *TSC2* they were c.1600-14C>T, c.2546-12C>T, c.3544A>G, c.5202T>C and c.5397G>C.

4.3 Treatment regimens

There were 6 (13.6%) patients who were not on any drug treatment. Two of these had never experienced seizures and four patients had been seizure free for a long time. Three patients were on antihypertensive (Captopril) treatment in addition to antiepileptic drugs and one patient used deperiprone for iron chelation as he also suffered from β Thalassemia major. Following is the breakup of the pattern of drug treatment in this study population.

TABLE 12: Treatment pattern in the study population

Treatment	Number of patients	%
Monotherapy (On one drug)	6	13.6
Polytherapy 2 drugs	18	40.9
3 drugs	9	20.4
4 drugs	4	9.0
5 drugs	1	2.2
Other drugs Antihypertensives	3	6.8
Iron chelation	1	2.2

TABLE 13: Drugs used in managements of the patients with TSC

Name of the drug	Number of patients
Sodium Valproate	19
Carbamazepine	14
Topiramate	14
Clobazame	13
Vigabatrin	11
Lamotrigine	3
Captopril	3
Deperiprone	1

Commonly used drugs for management of these patients are noted above in Table 13

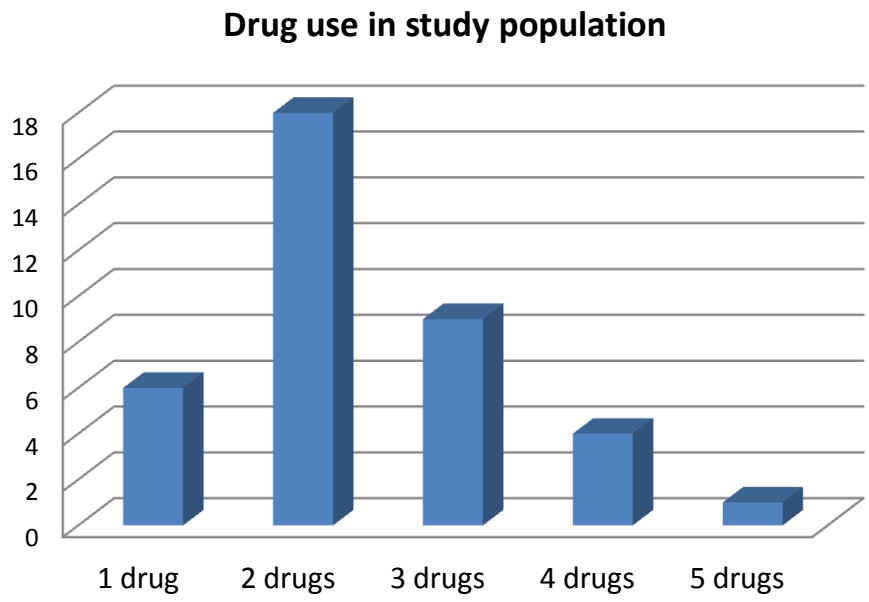


Fig 23: Drug treatment pattern in the study population

5. Discussion

Tuberous Sclerosis Complex (TSC) is an autosomal dominant disorder characterized by development of hamartomas in almost all organ systems of the body. Other characteristic features of TSC are epilepsy, intellectual disabilities, autism spectrum disorders and organ failure [62]. TSC is no more considered a rare genetic disorder with a birth incidence of 1/6000 to 1/10,000 and a population prevalence of 1 in 20,000. It is estimated that nearly one million individuals worldwide are affected with TSC, involving all ethnic groups irrespective of their gender [2, 27].

It is difficult to evaluate TSC clinically due to its highly variable phenotype with multisystem involvement and age dependent appearance of some clinical features. Even though the inheritance is autosomal dominant, about two- thirds of the cases are sporadic [62].

It is relatively challenging to diagnose TSC genetically due to the large size of the two genes responsible for TSC, with highly heterogeneous mutations scattered all over the two genes, the *TSC1* and *TSC2*. In this study we have used Sanger sequencing of all the exons in both genes as the first line of molecular testing to evaluate point mutations and small deletions, insertions and duplications. In failing to detect the above, MLPA has been done to look for large deletions or insertions. These two methods provide a sensitive strategy in molecular diagnosis of TSC.

During the last two decades, there has been a steady increment in understanding the phenotypic and genotypic variation associated with TSC. Knowledge has expanded to understand the tumor suppressor effects of TSC genes on the mTOR pathway, seeking for new treatment options for the disease [69, 70]. This study is an initial attempt to look at the possible genotypic composition of a cohort of patients from Sri Lanka with regard to TSC.

5.1 The phenotype and genotype of patients with TSC in Sri Lanka

The most common skin manifestation was hypopigmented macules found in 88.6%, followed by facial angiofibromas (47.7%), fibrous cephalic plaques (34%) and shagreen patches (29.5%). No confetti skin lesions were noted in the study group. These findings were similar to the published data except for facial angiofibromas and shagreen patches which were less prevalent in this population [2, 3, 28]. Among 44 patients, 39 (88.6%) suffered from epilepsy. In this study, evidence of infantile spasms (IS) was noted in 19 (43.1%), focal seizures (FS) in 36 (81.8%) and intractable epilepsy noted in 17 (38.6%) patients is comparable to previously reports [44]. Only five patients were seizure free and six patients had total seizure control with monotherapy, making the total of 25% of the patients who had seizure control. This is lower than reported other cohorts [44]. This may be due to sample selection bias, as the patients were recruited only from the tertiary care centers, where only the difficult to manage patients end up. There were three patients with a family history of epilepsy, but no family history of TSC. This might be due to sub-clinical TSC running in the family, which remains to be discovered.

When considering the major clinical criteria diagnosed by neuro-imaging techniques, cortical tubers were present only in 28 patients (63.6%), which is lower than compared to other reported data [2]. MRI is the procedure of choice in diagnosing brain manifestations in TSC and this technique more accurately localizes cortical tubers [71]. In this study MRI were available in only 17 patients and 14 of them reported presence of cortical tubers. Subependymal nodules (SEN) were reported in 30 patients (68.1%) which is also less than what reported previously [2], though it is reported that CT scanning is more accurate in detecting calcified SEN [71]. Among the 25 patients with available CT scans, 19 showed calcified SEN. There were seven patients with no available imaging reports, and these patients may have been added on to the presence of tubers and SEN if they were available.

Subependymal giant cell astrocytomas (SEGAs) were reported in 5 – 15% of the patients with TSC [2, 27] and the findings were similar in this study population.

A study reported that 55% of a children cohort with TSC with a mean age of 6.9 years had some renal abnormality [72]. In this study population (mean age 6.5 years) only 12 patients (27.3%) had renal angiomiolipomas, renal cysts or both.

Presence of lymphangioleiomyomatosis (LAM) was not investigated as this was a paediatric population and the only patient who was 18 years was a male.

According to previously reported data approximately 30% of the TSC mutations are familial, while the remaining 70% are *de novo* [8]. In this study 38.6% of the patients had a family history of TSC where one or more family members were clinically affected. Genotyping the parents may confirm a higher percentage of familial TSC in this population. Among nine patients with *TSC1* mutations, 55.5% had clinical familial history. In patients with *TSC2* mutations, only 33.3% were found with familial mutations. Hence, sporadic mutations are more often reported in *TSC2* mutations than with *TSC1* mutations. This agrees with the previous study that there is relative paucity of *TSC1* mutations in sporadic TSC when compared to *TSC2* mutations [73].

5.2. Pattern of mutations

To determine the pattern of mutations, the classification system for sequence variants at Ullevaal hospital (Appendix 5) was taken into account.

Classification system for sequence variants

This tool is being used at the department of Medical Genetics, University hospital, Ullevaal, Norway to classify mutations according to the possibility of being pathogenic. In this classification system, word pathogenic is referred to destructive for protein production and/or function. There are 5 categories in this classification system. The variants can be checked with databases, available literature and reported data with population based studies for

categorization. The well documented normal variants fall into category 1 and well documented pathogenic mutations fall into category 5. The uncertain variants are categorized into other 3 categories, depending on the evidence based manner, the most possible normal variants fall into category 2, while most possible pathogenic variants fall into category 4. The category 3 is the variants of uncertain significance (VUS) with lack of documentation. These are the most unpredictable variants that need functional studies to determine the exact effect caused by the mutation. This classification system is a reliable method to gain an idea of the novel mutations. The classification is developed by the expertise at the University hospital, Ullevaal, Norvay.

There were nine identified neutral variants which had been reported in previous studies as well as in databases. These normal variants fall into category 1 according to the classification. These variants were found in more than 6 patients in the study, yet it can be confirmed by checking with normal Sri Lankan population as the reported data are from different populations.

There were eight variants falling into category 2. As there is no reported data to confirm these are normal variants, it will be interesting to check with normal Sri Lankan population for confirmation.

The number of novel mutations was 22. Among them there were three missense mutations and a splice site mutation which falls into category 3, the variants of uncertain significance (VUS). The patient with deletion in *TSC2* exon 1 up stream and three patients with one or few base pair deletions in *TSC2* also belong to category 3. In these eight mutations, a further study into mRNA and protein functional status is needed to come to a conclusion, though the patients exhibit a severe clinical phenotype of TSC. Reporting of the same mutations resulting in TSC phenotype can add on more power to confirm these mutations as pathogenic.

There are 4 nonsense mutations and 4 frame shift mutations that can be classified as category 4. The four patients with large deletions in *TSC2* involving at least one exon and the patient with large deletion in *TSC1* also belong to category 4 as the predicted protein truncation effect is high with these mutations [29]. The only duplication reported is predicted to result in truncated protein, 42 amino acids downstream to the mutation in exon 33. Although these variants are completely novel, it is highly likely that they are pathogenic. Yet, it will be interesting to study protein functions to verify them as protein truncating mutations. Reported data on same mutation presenting in TSC phenotype will add on power to confirm these as pathogenic mutations.

The remaining 20 mutations are well documented pathogenic variants, which fall into category 5. Among them there are two patients with contagious gene syndrome, involving total deletion of *TSC2* and *PKD1* genes. The extent of the deletions are yet unknown. They might contain other neighboring genes such as *NTHL1* and *SLC9A3R2* in the deleted region. It will be interesting to perform a karyotyping followed by break point analysis in these two patients to look for the exact site of deletion and the genes involved.

The most common mutation in this study population is *TSC2* Ex38 c.5024C>T missense mutation reported in 3 families. These families are all from western province, Colombo district. There is no strong evidence to confirm that this is a founder mutation. However this can be studied by performing haplotype analysis of the nearby region. There are two other mutations in *TSC1* that are present in two families, a nonsense mutation and a missense mutation which are found in different ethnic groups. They carry a remote possibility of being founder mutations in Sri Lankan patients with TSC.

5.3. Infantile Spasms and genotype – phenotype correlation

Among the 19 patients with IS, 16 patients (84.2%) were found to have mutations in *TSC2* while two patients (10.5%) had mutations in *TSC1* and one patient (5.3%) had no mutations

in either gene. In this study, a relatively high percentage of *TSC2* mutations are associated with IS when compared to previously reported data [29]. As our sample size is small, this might be coincidental. Only one of the patients with IS in our cohort had a missense mutation in the central domain of *TSC2* (exon 23 – 33), which is less than expected, if the location of the mutation were to be unconditionally spread throughout the gene (p=0.052). This agrees with the findings of van Eeghen and colleagues, which show that missense mutations in the central *TSC2* are less likely to cause IS [29]. Focal epilepsy (FE) was reported in 89.4% of the patients who had IS, and 47.3% of them were resistant to drugs. This reveals that the patients with IS and IS together with FE are more prone to be resistant to drugs [44]. 98.1% of the children who are resistant to antiepileptic drugs have had IS together with FS and 88.2% experienced the first episode of convulsion during the first six months of their life. This confirm that IS is a risk factor for intractable epilepsy and that seizure onset within the first year of life with IS and/or focal seizures is characteristic of drug resistant epilepsy in patients with epilepsy [43, 44].

In three patients (6.8%), initial presentation was febrile convulsions, and these patients later developed IS. TSC presenting with febrile convulsions as the first episode of seizures has been reported previously [74], but the association to the development of IS has not been described. To establish whether febrile convulsions are a presenting feature of TSC with IS, further investigation is needed.

5.4. *PKD1* deletions

According to published data, 2% of the patients with TSC are found to be with contagious gene deletions syndrome which includes the deletion of whole or part of the *PKD1* gene located only 48 base pairs away from *TSC2* gene on chromosome 16p13.3 region [27]. In this study population, 3 (6.8%) patients were found with *TSC2* and *PKD1* deletion, whereas 2 of

these (4.5%) had complete deletion of *TSC2*, resulting in haplo-insufficiency of *TSC2*. In addition to these two patients, patients with missense mutations and deletions in the GAP domain (exon 34-38) of the *TSC2* gene (as described by Maheshwar et al [75]), lead to grade 3 or grade 4 renal manifestations. The relationship between mutations in GAP domain of *TSC2* and renal involvement of TSC is yet mainly unknown.

5.5. Overall genotype

The Sri Lankan population with TSC is relatively similar with regards to genotype and phenotype to the other reported patients with TSC. The frequencies of different types of mutations in *TSC1* and *TSC2* are mainly as reported in other studies [22], and the findings of this study is shown in figure 19.

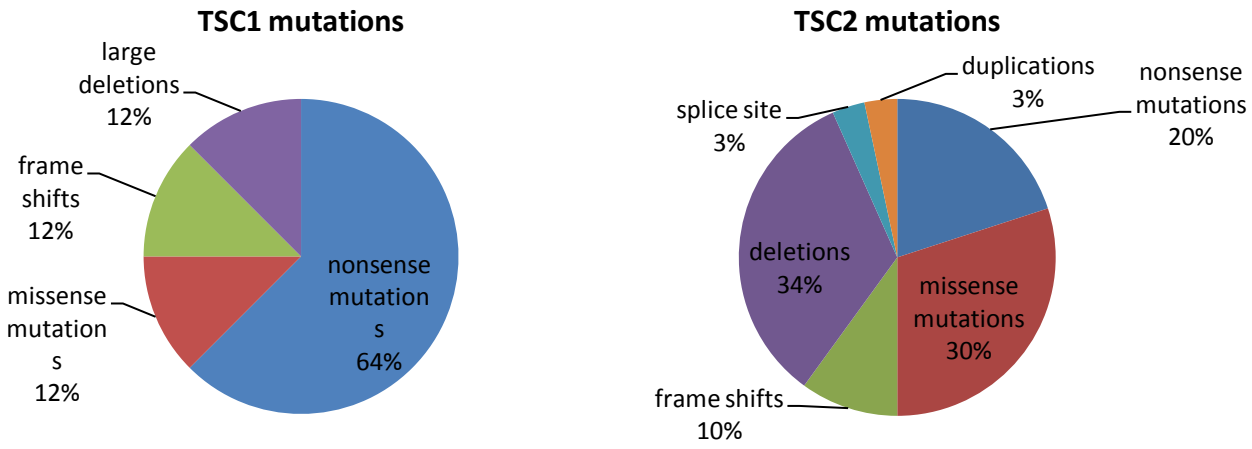


Fig 24: Frequencies of different types of mutations found in the study

There are 6 patients with no variants in either *TSC1* or *TSC2* genes. The cause of TSC in these patients is so far not known. Could there be a third locus giving rise to the TSC phenotype? If so, that hitherto unknown gene should probably be acting on the mTOR signaling pathway, in order to give rise to the same phenotype. This is an interesting area to be explored. We have only sequenced the coding regions of *TSC1* and *TSC2*. May be the disease causing mutations are within one of the genes, but in an intronic region, promoter

region or a regulator region as there are well documented pathogenic mutations reported in non-coding regions causing TSC [76]. Another possible explanation at least in the sporadic cases, is somatic mosaicism that can be missed by testing only the peripheral lymphocytes [77, 78].

Patient 13 is diagnosed β- thalassemia major apart from his novel *de novo* mutation in *TSC1* exon 18, c.2356C>T which is a nonsense mutation that fall into category 4. There are around 2000 reported patients with β- thalassemia major in Sri Lanka [79], therefore the estimated prevalence is 1 in 10,000 individuals. Considering this high predicted prevalence, this can be concluded as an incidental event.

6. Conclusion

Clinical characteristics of TSC in Sri Lankan children were similar to the previously described features of the TSC phenotype. There were 22 novel mutations in the *TSC1* and *TSC2* genes reported in this study population. Among them, 14 mutations had a high potential of being pathogenic. There were six patients with TSC phenotype, but where no mutations were found in coding regions of either gene, which is opened for further investigation.

7. Limitations of the Study

The sample population was gathered from tertiary care centers around the country, basically on referrals. As only the severely affected patients encounter these centers, the patients who were not followed up by these clinicians have been missed. Therefore selection bias is the major limitation in this study.

Psychiatric assessment has not been done routinely at the time of diagnosis for the patients with TSC in Sri Lanka. Even though cognitive disability and neuropsychiatric phenotype is common in TSC, neurobehavioral phenotype could not be investigated.

Parents of most of the patients with novel mutations were not genotyped. It will be interesting to genotype the parents to obtain the inheritance pattern of novel mutations.

Only the coding regions were sequenced in *TSC1* and *TSC2*. Therefore mutations in noncoding regions, promoter regions, modulators and enhancers are being missed.

8. Recommendations

At the beginning of this study, pre test genetic counseling was offered to all the parents of the participants by the principal investigator. During that time only eight parents knew the genetic factor of TSC. It will be much easier for post test counseling as there is some prior knowledge regarding the genetic predisposition among the parents. Genetic counseling will be a benefit to the patients as an outcome of this study.

MRI is an important investigation to diagnose cortical tubers, SEN and SEGA which are hallmarks in clinical diagnosis of TSC [71]. According to recommendations of the international TSC consensus conference 2012, every patient should undergo MRI of the brain at the time of establishment of the diagnosis of TSC [1]. It is recommended to have at least one MRI scan, probably at the time of diagnosis for every patient with TSC in Sri Lanka, though the resources are limited.

Vigabatrin is considered the drug of choice for IS in TSC [80]. Only 11 patients out of 19 are being treated with Vigabatrin. This is due to unavailability of the drug in centers other than the Lady Ridgeway Hospital for children. If the drug can be made available, the patients with IS will benefit from this. The mTOR inhibitors are not available in Sri Lanka yet. Apart from patients with epilepsy and cognitive impairment, patients with SEN, SEGA and renal angiomyolipomas could benefit from this drug [81, 82]. This will help reduce the polytherapy and control intractable seizures. Patients with facial fibromas will also benefit with systemic or topical application of mTOR inhibitors [83].

For the patients with drug resistant epilepsy, modified ketogenic diet is a good option [27], though it is difficult to offer this diet for the children with intractable seizures, as it needs close monitoring at dietitians' departments. It is worthwhile to use this diet as it may prevent polytherapy while gaining better seizure control.

Vagal nerve stimulation is considered in TSC patients with drug resistant epilepsy, where the surgical management is not feasible [84]. Vagal nerve stimulation is not a good choice in Sri Lanka, as the high expense might be unbearable for most patients. If it can be implemented through the Government and offered free of charge, this will benefit the patients who need this method of treatment.

9. Future Prospective

Next generation sequencing is a technique that can sequence large regions of DNA at a much lower cost than Sanger sequencing. Using this technique in diagnostics, one might consider to sequence the complete region of the gene, including intronic regions, promoters, regulatory elements and untranslated regions (UTR) [85]. This might also be used to sequence the patients where no mutations in coding regions of *TSC1* and *TSC2* are found, as one might suspect that there are mutations outside the coding part of the genes that causes TSC in these patients.

As there are 22 novel mutations it could be interesting to do mRNA studies to investigate the transcripts of and different transcripts of *TSC1* and *TSC2* in the patients. Furthermore, it could be beneficial to perform functional studies to determine the effect of novel mutations. i.e to support or exclude pathogenicity of the mutations.

Development of new databases with variant data for South Asian population will be beneficial for researches, geneticists and patients, as it will improve the variant evaluation and the classification of the variants in both research and diagnostics.

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11.Appendixes

Appendix 1: List of abbreviations

TSC – Tuberous Sclerosis Complex

TS – Tuberous Sclerosis

TSC1 – Tuberous Sclerosis Complex 1 gene

TSC2 – Tuberous Sclerosis Complex 2 gene

mTOR – mammalian Target Of Rapamycin

GTP – Guanidin Triphosphate

Rheb – Ras homolog enriched in brain

MRI – Magnetic Resonance Image

SEN – Subependymal Nodules

SEGA – Subependymal Giant cell Astrocytomas

IS – Infantile Spasms

EEG – Electroencephalogram

PKD1 – polycystic kidney disease 1 gene

ADPKD – Autosomal Dominant Polycystic Kidney Disease

LAM – Lymhangioliomyomatosis

CR – Cardiac Rhabdomyomas

AH – Astrocytic Hamartoma

TAND –TSC-Associated Neuropsychiatric Disorder

GFR – Glomerular Filtration Rate

CT – Computed Tomography

HRCT – High-Resolution Computed Tomography

ACTH – Adrenocorticotropin Hormone

ECG – Electrocardiogram

DNA – Deoxyrhybo Nucleic Acid

mRNA – messenger Rhybo Nucleic Acid

LRH – Lady Ridgeway Hospital for Children

ILAE – International League Against Epilepsy

USS – Ultra Sound Scan

2D echo – Two Dimensional echocardiogram

PCR – Polymerase Chain Reaction

dNTPs – deoxynucleotide Triphosphates

SNV – Single Nucleotide Variation

MLPA – Multiplex Ligation dependent Probe Amplification

CNV – Copy Number Variation

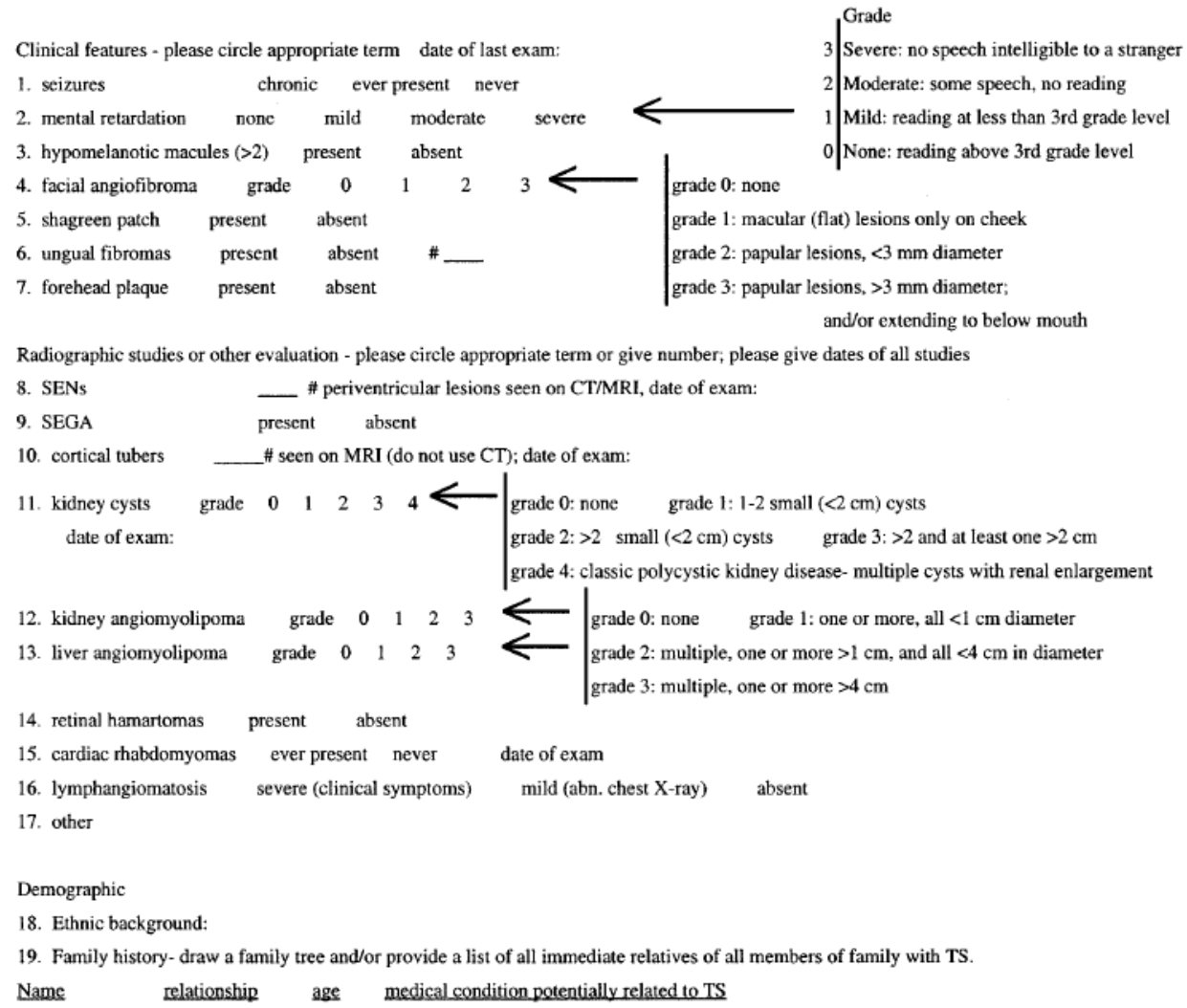
VUS – Variants of Uncertain Significance

FE – Focal Epilepsy

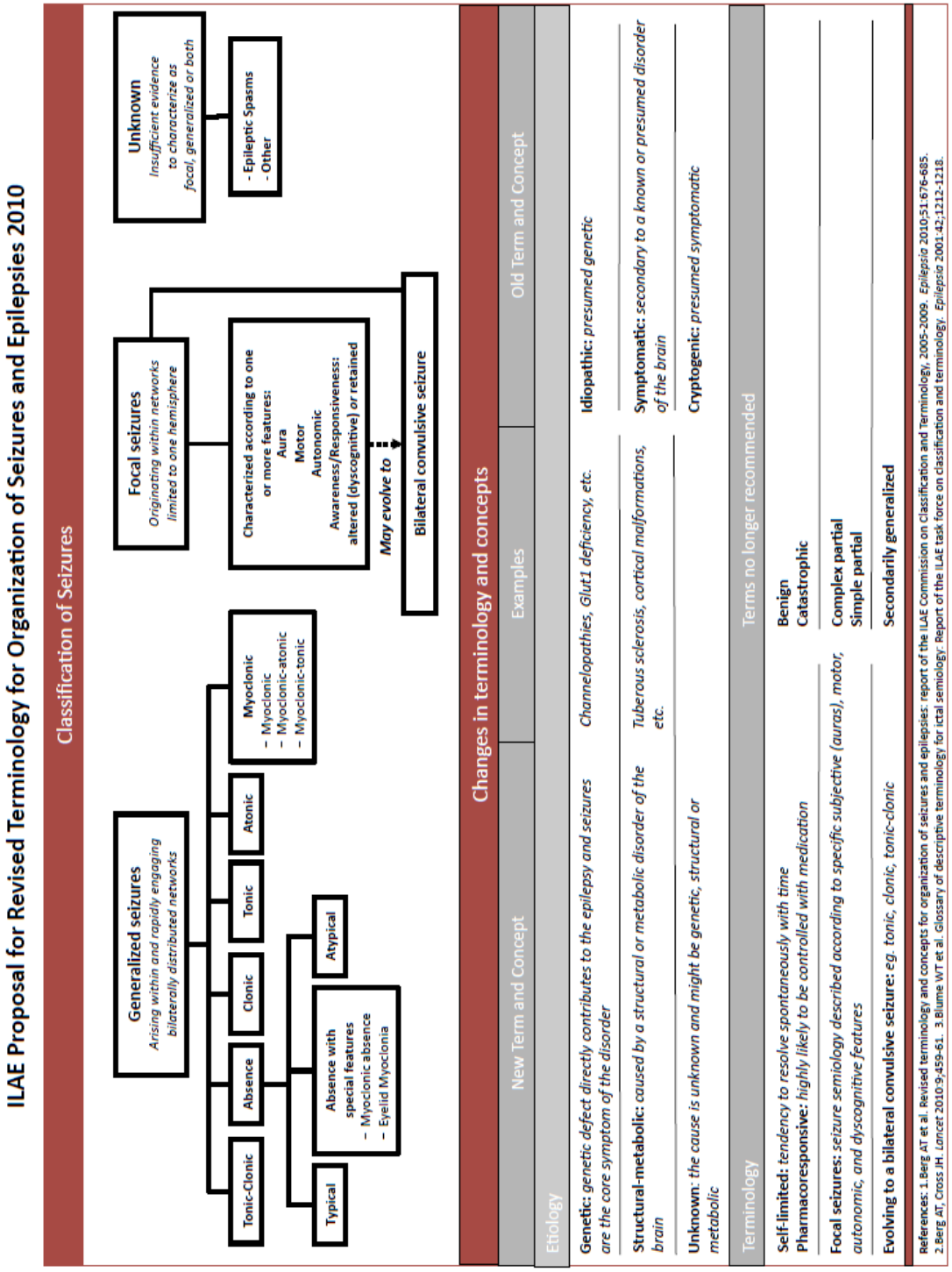
UTR – Untranslated Regions

Appendix 2: Clinical Diagnostic Criteria

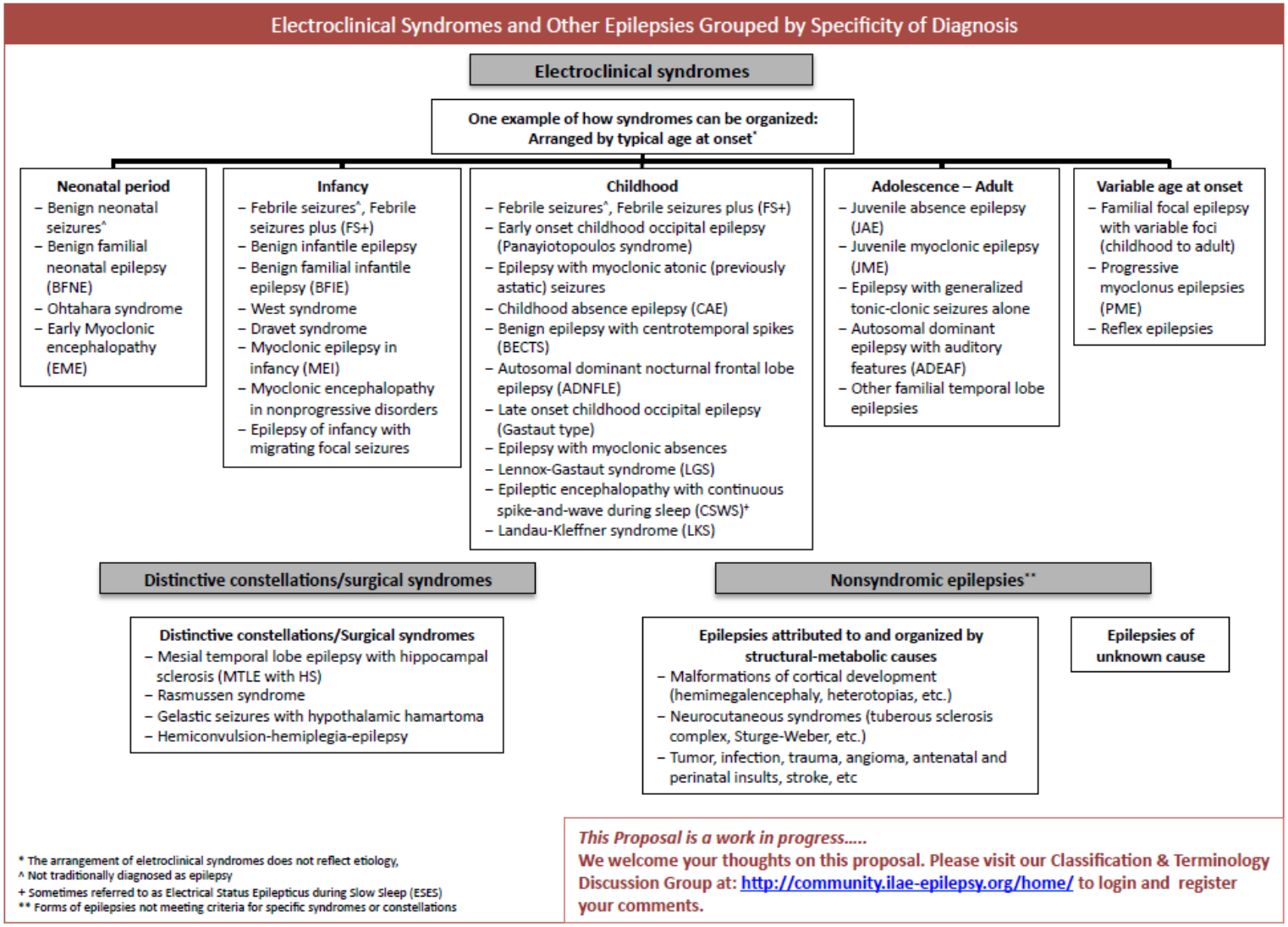
Grading of clinical manifestations



International League Against Epilepsy (ILAE) Classification 2010



ILAE Proposal for Revised Terminology for Organization of Seizures and Epilepsies 2010



Appendix 3: Documents used for subject recruitment

This appendix contains the English, Sinhala and Tamil versions of the documents which were used for subject recruitment

1. Information leaflet for the parents/guardians of the study participants used to recruit patients with Tuberous Sclerosis Complex
2. Consent form used for recruitment patients with Tuberous Sclerosis Complex
3. Data Collection booklet (Only in English, as it was filled by the principal investigator)

INFORMATION SHEET

A STUDY OF TUBEROUS SCLEROSIS IN A COHORT OF SRI LANKAN PATIENTS

This study is conducted by me, Dr. Samudita Senaratne, Msc student in Clinical Genetics at Human Genetics Unit, Faculty of Medicine, University of Colombo. I would like to invite you to take part in the research study titled "A STUDY OF TUBEROUS SCLEROSIS IN A COHORT OF SRI LANKAN PATIENTS". This study is conducted in collaboration with University of Oslo, Norway by me, under the supervision of

Prof. Vajira H.W. Dissanayeke, at the Human Genetics Unit, University of Colombo

Dr. Karl Otto Nakken, at the National Center for Epilepsy, Norway

Dr. Jithangi Wanigasinghe, at the Department of Pediatrics, Faculty of Medicine, University of Colombo and

Dr. Dulika Sumathipala, at the Human Genetics Unit, University of Colombo.

1. Purpose of the study

Genes determine how we look and also determine our body functions. Genetic information in our body is carried by genes which contain in structures called chromosomes within the nucleus of the cell. When there are abnormalities in genes it might lead into genetic disorders that run along generations in a family.

Tuberous Sclerosis Complex (TSC) is a genetic disorder that causes noncancerous tumors in many different organs, namely in the brain, skin, eyes, heart, kidney and lungs. The aspects of TSC that most strongly affect quality of life are generally associated with brain including fits, delay in development, reduced thinking ability and a psychiatric illness called autism. However many people with TSC are living independent and virtually healthy lives. The incidence and severity of the various aspects of TSC can vary widely between individuals, even between identical twins.

Research into genetic defects of TSC patients in Sri Lanka has not been done before. Therefore a need to determine genetic defect of Sri Lankan patients with TSC arises.

Purpose of the study is to look at the different presentations of the patients with Tuberous Sclerosis Complex (TSC) to get an idea of the different presentations of the disorder in the Sri Lankan children. Gene testing will be done to detect the genetic defect and to relate it with the disease features of the child.

2. Voluntary participation

Taking part of your child in this study is voluntary. You are free to not add your child at all or to withdraw from the study at any time despite consenting to take part earlier. There will be no loss of medical care or any other available treatment for your child's illness or condition to which he/she is otherwise entitled. If you decide not to participate you may withdraw him/her from the study at any time by informing us.

3. Duration, procedure of the study and participant's responsibilities

The study will be conducted over 1 year. We require your permission to ask you questions, examine your child, have access to his/her medical records such as the clinic book, and investigation reports. We also need your permission to publish the data collected in a scientific journal. We will not mention your child's name or any other information that your child could be identified when we publish the results. We may also need to take 5ml of venous blood from your child to do the genetic testing. After testing for the two genes which are responsible for TSC, if there is left out blood samples it will be stored at -80C for further research into TSC in Sri Lanka and kept at least for a time period of ten (10) years.

4. Potential Benefits

Taking part in this study will help you to know the cause or genetic defect that has made your child develop Tuberous Sclerosis Complex. This will contribute to the increasing of knowledge about Tuberous Sclerosis Complex in Sri Lankan patients. It will help us to come to a genetic diagnosis. It will contribute to the knowledge of the disorder in Sri Lankan patients.

5. Risks, hazards and discomforts

Blood will be drawn from your child to detect the genetic defect causing Tuberous Sclerosis. Approximately 5ml of blood will be taken for testing from your child. The risk to your child by The electronic database containing the data will have only the subject study number. The computer containing the database would be password protected. Participating in the study is the risk of pain, bruising and infection at the needle prick site. These will be minimized by performing blood drawing under aseptic conditions by trained nurse or a medical technician specialized in blood drawing (phlebotomist).

6. Reimbursements

There will be no payment for you or your child for participating in the study, but you will be given a copy of the molecular genetic test results done on your child.

7. Confidentiality

Confidentiality of all records is guaranteed. Information that you or your child can be identified will not be released or published**.** The data collection booklet is designed to ensure confidentiality of information gathered. These data will never be used in such a way that you or your child could be identified in any way in any public presentation or publication without your permission.

8. **Termination of study participation**

You may withdraw participation of your child in this study at any time, with no penalty or effect on medical care or loss of benefits. Please notify us as soon as you decide to withdraw your consent. However it will not be possible for you to withdraw once the results are sent for publication or once the results are published.

9. **Clarification**

If you have questions about any of the tests / procedures or information please feel free to ask any of the persons listed below by calling 011 2689 545.

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Tel: 077 7697 480
011 3165 805

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Medical Geneticist
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011 2689 545

ටියුබරස් ස්ක්ලෙරෝසිස් කොම්ප්ලෙක්ස් සහිත ශ්‍රී ලාංකීය රෝගීන් පිළිබඳ අධ්‍යයනය

කොළඹ වෛද්‍ය පීඨයේ මානව ප්‍රවේණි විද්‍යා අංශයේ පශ්චාත් උපාධියක් හදාරන වෛද්‍ය සමුදිතා සේනාරත්න වන මම ඇතුලු අනෙකුත් පර්යේෂණ සාමාජිකයින් වන

මහාචාර්ය වජිර එච්. ඩබ්. දිසානායක - මානව ප්‍රවේණි විද්‍යා අංශය, වෛද්‍ය පීඨය , කොළඹ.

වෛද්‍ය කාල් ඔටෝ නකෙන් - අපස්මාරය පිළිබඳ ජාතික ආයතනය , නොර්වේ.

වෛද්‍ය ජිතාන්ගී වනිගසිංහ - ළමා රෝග අංශය, වෛද්‍ය පීඨය , කොළඹ.

වෛද්‍ය දුලීකා සුමතිපාල - මානව ප්‍රවේණි විද්‍යා අංශය, වෛද්‍ය පීඨය , කොළඹ.

යන අය විසින් කරනු ලබන ඉහත සඳහන් පර්යේෂණයට සහභාගී වීම සඳහා ඔබට ආරාධනා කිරීමට අපි කැමැත්තෙමු. මෙම අධ්‍යයනය කොළඹ වෛද්‍ය පීඨය හා නොර්වේහි පිහිටි ඔස්ලෝ විශ්වවිද්‍යාලය අතර සහයෝගිතාවයෙන් සිදු කරනු ලැබේ.

1.මෙම අධ්‍යයනයේ අරමුණ

අපගේ හැඩරුව හා ශරීරයේ සියළුම ක්‍රියාවන් පාලනය කරනුයේ ජාන මගිනි. මෙම ජාන අපගේ සෛලවල න්‍යෂ්ටියේ ඇති වර්ණදේහවල පිහිටා ඇත. මෙම ජාන මගින් එක් පරම්පරාවක ලක්ෂණ තවත් පරම්පරාවකට ගෙනයාම ප්‍රවේණිය නම් වේ. ඇතැම් රෝගද ප්‍රවේණිගත (ආරයට යන රෝග/ පරම්පරාවට යන රෝග) විය හැක.

ටියුබරස් ස්ක්ලෙරෝසිස් කොම්ප්ලෙක්ස් යනු ජාන වල හටගන්නා වෙනස්කම් (ජාන විකෘති) නිසා හටගන්නා රෝගයකි.මෙහි ප්‍රධාන ලක්ෂණය වනුයේ මොලය, සම, ඇස, හෘදය, වකුගඩු හා පෙනහලු වැනි අවයව වල හටගන්නා පිළිකාමය නොවන අස්වාභාවික සෛල වර්ධනයකි (ගෙඩි). මේ අතරින් නීරෝගී දිවිපැවැත්ම සඳහා වැඩි වශයෙන් භාදා වනුයේ මෙම රෝගය මොළය හා සම්බන්ධව හටගත් විටයි. මෙහිදී වලිප්පුව, කායික හා මානසික වර්ධනයේ ඌනතා හා ඕටිසම්වැනි මානසික රෝගී තත්වයන් ඇති විය හැක. කෙසේ වුවද ටියුබරස් ස්ක්ලෙරෝසිස් කොම්ප්ලෙක්ස් සහිත බොහෝ රෝගීන් ස්වාධීන හා නීරෝගී ජීවිත ගත කරති. මෙම රෝගයේ අවදානම් ස්වභාවය සහ රෝග ලක්ෂණ පුද්ගලයන් අතර විවිධ මට්ටම් වලින් දැකිය හැකි අතර රෝගයේ තීව්‍ර භාවය සම නිවුන් දරුවන් අතර පවා වෙනස් විය හැක.

ශ්‍රී ලංකාවේ ටියුබරස් ස්ක්ලෙරෝසිස් කොම්ප්ලෙක්ස් රෝගයට හේතුවන ජානමය වෙනස්කම් පිළිබඳව මීට පෙර පර්යේෂණ සිදුකර නැත. එමනිසා ටියුබරස් ස්ක්ලෙරෝසිස් කොම්ප්ලෙක්ස් සහිත ශ්‍රී ලාංකීය රෝගීන් සඳහා බලපවත්නා ජාන විකෘතිතා හඳුනා ගැනීමේ අවශ්‍යතාව මතු වී තිබේ.

මෙම පර්යේෂණයේ ප්‍රධාන අරමුණ වනුයේ ටියුබරස් ස්ක්ලෙරෝසිස් කොම්ප්ලෙක්ස් සහිත ශ්‍රී ලාංකීය රෝගීන්ගේ රෝග ලක්ෂණ වල විවිධත්වය අධ්‍යයනයයි. එම රෝගීන්ගේ රෝග තත්ව සහ ජාන අතර සම්බන්ධතාවය පරීක්ෂා කිරීම අනෙක් අරමුණ වේ.

2. ස්වෙච්චා සහභාගීත්වය

මෙම අධ්‍යයනය සඳහා ඔබගේ දරුවා සහභාගී කරවීම ඔබගේ කැමැත්තෙන්ම සිදු කරන්නෙකි. මෙයට ඔබගේ දරුවා සහභාගී නොකිරීමට ඔබට පූර්ණ අයිතිය ඇති අතර සහභාගී වීමට කලින් කැමැත්ත පල කර තිබුනද ඕනෑම අවස්ථාවක අප හට දැනුම්දී ඉන් ඉවත් වීමට පූර්ණ අයිතිය ඇත. එමගින් දරුවාට ලබාදෙන ප්‍රතිකාර වල කිසිදු වෙනසක් සිදු නොවේ.

වසර දහය (10)ක පමණ කාලයක් සෙල්සියස් අංශක -80 උෂ්ණත්ව තත්ව යටතේ එම සාම්පල සුරක්ෂිතව තබා ගනු ලැබේ.

3. කාල සීමාව, පර්යේෂණ ක්‍රියාවලිය සහ සහභාගී වන්නන්ගේ වගකීම්

අධ්‍යයන කාලසීමාව වසරක් පුරා විහිදේ. මෙම පර්යේෂණයට ඔබගේ දරුවා සහභාගී කරවීමට ඔබ කැමති නම් ඔබගෙන් ප්‍රශ්න ඇසීමට, ඔබගේ දරුවා පරීක්ෂා කිරීමට හා ඔහුගේ / ඇයගේ වෛද්‍ය වාර්තා පිරික්සීමට අප හට සිදු වේ.පරීක්ෂණයේ ප්‍රතිඵල විද්‍යාත්මක සඟරා වල පල කිරීමටද ඔබගේ අවසරය අවශ්‍ය වේ. එසේ පල කිරීමේදී ඔබගේ දරුවාගේ නම හෝ වෙනෙත් හඳුනා ගත හැකි අන්දමේ කිසිදු තොරතුරක් අප විසින් සපයන්නේ නැත. ජාන පරීක්ෂණ සඳහා මි.ලී.5ක පමණ රුධිර සාම්පලයක් ඔබගේ දරුවාගේන් ගැනීමට සිදුවනු ඇත. ටියුබරස් ස්ක්ලෙරෝසිස් කොම්ප්ලෙක්ස් හා සම්බන්ධ ජාන සඳහා පරීක්ෂා කිරීමෙන් අනතුරුව රුධිර සාම්පලය ඉතිරි වුවහොත්, මෙම රෝගය හා බැඳුනු ඉදිරි පර්යේෂණ කටයුතු සඳහා වසර 10 ක කාලයක් සුරකිව තබාගනු ලැබේ.

4.මින් ලද හැකි ප්‍රතිලාභ

මෙම පරීක්ෂණයට සහභාගී වීමෙන් ඔබට, දරුවාගේ ටියුබරස් ස්ක්ලෙරෝසිස් කොම්ප්ලෙක්ස් රෝග තත්වයට හේතු සාධක වූ ජානමය අක්‍රමිකතාව දැනගත හැකි වනු ඇත. ලාංකීය ජනතාව අතර පවතින රෝගය හා බැඳුනු ජානමය අක්‍රමිකතාවයන් පිළිබඳවද එමගින් අදහසක් ගත හැකි වනු ඇත.

5.අවදානම්,අනතුරු සහ අපහසුතා

ටියුබරස් ස්ක්ලෙරෝසිස් කොම්ප්ලෙක්ස් රෝගයට තුඩුදුන් ජානමය අක්‍රමිකතාව දැනගැනීමට ඔබගේ දරුවාගේන් මි.ලී.5ක රුධිර සාම්පලය ලබා ගැනීමේදී ඔබගේ දරුවාට යම් අපහසුතාවක් ඇතිවිය හැක. කලාතුරකින් රුධිර සාම්පලය ලබා ගැනීමේදී එන්නත් කටුව නිසා යම් තැල්මක්, ඉදිමීමක්, ආසාදනයක් එම ස්ථානයේ හට ගත හැක. මෙම තත්වයන් අවම කර ගැනීම සඳහා රුධිර සාම්පලය ලබා ගැනීම සියලු ආරක්ෂිත තත්වයන් යටතේ පළපුරුදු හෙද නිලධාරිනියක් මගින් සිදු කරනු ලැබේ.

6.දීමනා

මෙම අධ්‍යයනයට සහභාගී වීම වෙනුවෙන් ඔබට හෝ ඔබගේ දරුවාට කිසිදු දීමනාවක් නොලැබේ. එහෙත් ඔබගේ දරුවාගේරෝග තත්වය පිළිබඳ පැහැදිලි වැටහීමක් ලැබෙන අතර තම දරුවාගේ ජානමය අක්‍රමිකතාව පිළිබඳ වාර්තාවක් ලැබේ.

7.රහසිගත බව.

සියලුම තොරතුරු සහිත වාර්තාවන් සහ අධ්‍යයනය මගින් ලබා ගන්නා දත්තයන්ගේ රහස්‍යභාවය තහවුරු කරන අතර, ඔබ හෝ ඔබගේ දරුවාගේ අනන්‍යතාවය හඳුනාගත හැකි ආකාරයේ කිසිවක් ඔබගේ කැමෙත්තකින් තොරව හෙළි කිරීමක්, ඉදිරිපත්කිරීමක් හෝ ප්‍රකාශයට පත් කිරීමක් සිදු කරනු නොලැබේ. දත්ත එකතු කිරීමේ පත්‍රිකාව සාකසා ඇත්තේද ඔබගේ රහස්‍යභාවය තහවුරු කෙරෙන අයුරෙනි. විද්‍යාත්මක සඟරාවක මෙම පර්යේෂණ වාර්තා පල කිරීමට අවශ්‍ය වූ විටද කිසිදු අයුරකින් ඔබගේ අනන්‍යතාවය හෙළි නොවන අයුරින් අපි එය පල කරන්නෙමු.

8. අධ්‍යයනයට සහභාගීවීම නැවැත්වීම.

අධ්‍යයනයට සහභාගීවීමට දුන් කැමත්ත ඉවත් කර ගැනීම අධ්‍යයනයේ කුමන හෝ අදියරකදී සිදු කිරීමට ඔබට හැක. එසේ සිදු කරන්නේ නම් එම තීරණය ගත් විගසම ඒ බව අපහට කරුණාකර දැනුම් දෙන්න. නමුත් එකතු කර ගන්නා ලද දත්ත ප්‍රකාශයට පත කිරීමෙන් පසුව ඔබට අධ්‍යයනයෙන් ඉවත් වීමට නොහැක.

9.වැඩිදුර තොරතුරු

ඔබට මෙම ක්‍රියා පටිපාටීන් පිළිබඳ කිසියම් ප්‍රශ්නයක් ඇත්නම් හෝ වැඩි දුර තොරතුරු අවශ්‍යනම් පහත සඳහන් වෛද්‍යවරුන් අමතන්න.

මහාචාර්ය වජිර එච්. ඩබ්. දිසානායක - මානව ප්‍රවේණි විද්‍යා අංශය, වෛද්‍ය පීඨය , කොළඹ. දූ.ක. 0112689545

වෛද්‍ය සමුදිතා සේනාරත්න - පශ්චාත් උපාධි අපේක්ෂිකා , මානව ප්‍රවේණි විද්‍යා අංශය, වෛද්‍ය පීඨය , කොළඹ. දූ.ක. 0777697480, 0113165805

தகவல் பத்திரம்

இலங்கையில் டியுபெரஸ் ஸ்க்லேரோசிஸ் (Tuberous Sclerosis) நோயினால் பாதிக்கப்பட்ட நோயாளிகள் பெருங்குழுவில் இந்நோயைப் பற்றிய ஒரு பெரும் ஆய்வு.

மனித மரபியல் பிரிவு, கொழும்பு மருத்துவ பீடத்தில் மருத்துவ மரபியல் முதுநிலை கல்வி மாணவியாக இருக்கும் Dr. சமுதிதா சேனாரத்ன ஆகிய நான் இந்த ஆய்வை மேற்கொள்கிறேன். " இலங்கையில் டியுபெரஸ் ஸ்க்லேரோசிஸ் (Tuberous Sclerosis) நோயினால் பாதிக்கப்பட்ட நோயாளிகள் பெருங்குழுவில் இந்நோயைப் பற்றிய ஒரு பெரும் ஆய்வு" எனும் தலைப்பில், மனித மரபியல் பிரிவு, கொழும்பு மருத்துவ பீடத்தில் நடைபெறும் இந்த ஆய்வில் பங்குபெற உங்களையும் அழைக்கிறேன். இந்த ஆய்வானது நோர்வேயிலுள்ள ஒஸ்லோ பல்கலைக்கழகதினுடைய ஒத்துழைப்புடன் பின்வருவோரின் மேற்பார்வையின் கீழ் நடைபெறுகிறது:

பேராசிரியர். வஜிர H.W. திசாநாயக (மனித மரபியல் பிரிவு, மருத்துவ பீடம், கொழும்பு பல்கலைக்கழகம்)

Dr. கார்ல் ஒட்டோ நக்கேன் (தேசிய வலிப்பு மையம்,நோர்வே)

Dr. ஜிதாங்கி வணிகசிங்க (குழந்தை மருத்துவப் பிரிவு, மருத்துவ பீடம், கொழும்பு பல்கலைக்கழகம்)

Dr. துலிகா சுமதிபால (மனித மரபியல் பிரிவு, மருத்துவ பீடம், கொழும்பு பல்கலைக்கழகம்)

இந்த ஆய்வின் குறிக்கோள்

எமது தோற்றங்களையும் உடல் செயற்பாடுகளையும் தீர்மானிப்பது மரபணுக்களாகும். இந்த மரபியல் தகவல்களைக் கொண்டிருக்கும் மரபணுக்களானது எமது கலக்கருவில் உள்ள நிறமூர்த்தங்களில் அடங்கியுள்ளன. இந்த மரபணுக்களில் மாற்றங்கள் ஏற்படும் போது பரம்பரையாக வரும் நோய்கள் ஏற்படலாம்.

டியுபெரஸ் ஸ்க்லேரோசிஸ் கொம்ப்ளெக்ஸ் (Tuberous Sclerosis Complex) ஒரு மரபியல் சார்ந்த நோயாகும். இது மூளை, தோல், கண்கள், இதயம், சிறுநீரகங்கள் மற்றும் சுவாசப்பைகளில் புற்றுப்பண்பு அல்லது வீரியம் அல்லாத கட்டிகளை உண்டாக்கும். இந்நோயுடன் தொடர்பான வாழ்க்கைத்தரத்தை அதிகமாகப் பாதிக்கும் அம்சங்களாவன வலிப்பு, பின்தங்கிய வளர்ச்சி, அறிவு வளர்ச்சிக் குறைபாடு, மனஇறுக்கம் போன்றவையாகும். இருப்பினும், இநோயால் பாதிக்கப்பட்ட பலர் சுதந்திரமாகவும் ஆரோக்கியமாகவும் வாழ்கின்றனர். இநோயின் தாக்கம் மற்றும் தீவிரம் இவ்வோருவரிடையே, ஒத்த இரட்டையர்களில் கூட மாறுபடலாம்.

இலங்கையில் டியுபெரஸ் ஸ்க்லேரோசிஸ் கொம்ப்ளெக்ஸ் நோயாளிகளில் காணப்படும் மரபியல் குறைபாடுகளைப்பற்றி இதுவரை எந்த ஆய்வுகளும் நடத்தப்படவில்லை. எனவே, இந்நோயாளிகளில் மரபியல் குறைபாடுகளை அடையாளம் காணுவது தேவையாக உள்ளது.

டியுபெரஸ் ஸ்க்லேரோசிஸ் கொம்ப்ளெக்ஸ் நோயினால் பாதிக்கப்பட்ட நோயாளிகளின் தோற்றவமைப்புக்களை கண்டறிந்து, இந்நோயின் வேறுபாடுகளைப் பற்றி அறிவதே இந்த

ஆய்வினுடைய நோக்கமாகும். மரபியல் குறைபாட்டினை கண்டறிய மரபியல் சோதனை செய்யப்பட்டு அதன் முடிவுகள் நோயாளியின் மருத்துவ அம்சங்களுடன் தொடர்புபடுத்தப்படும்..

தன்னார்வ பங்கேற்ப்பு

இந்த ஆய்வில் உங்கள் குழந்தையை பங்குபெறச்செய்வது உங்களது சுய விருப்பம். உங்கள் குழந்தையை நீங்கள் இந்த ஆய்வில் பங்கேற்காமல் செய்யலாம். முன்னர் சம்மதம் தெரிவித்திருந்தாலும் எவ்வேளையிலும் நீங்கள் உங்கள் குழந்தையை இந்த ஆய்விலிருந்து விலகிக்கொள்ள முடியும். இதனால் உங்கள் குழந்தைக்கு வழங்கப்படும் மருத்துவ கவனிப்போ, அவருக்கு உரித்தான எந்தவொரு சிகிச்சையோ நிறுத்தப்பட மாட்டாது. நீங்கள் இந்த ஆய்விலிருந்து விலகிக்கொள்ள முடிவெடுத்தால், எங்களுக்கு அறிவிப்பதன் மூலம் விலகிக்கொள்ளலாம்.

ஆய்வின் காலம், செயல்முறை மற்றும் பங்குபெறுவோரின் கடமைகள்.

இந்த ஆய்வு ஒருவருட காலத்திற்கு மேலாக நடத்தப்படும். உங்களிடம் கேள்விகள் கேட்பதற்கு , உங்களது குழந்தையை பரிசீலனை செய்வதற்கு, குழந்தையின் மருத்துவப் பதிவுகளை பார்வையிட்டு ஆராய்வதற்கு எங்களுக்கு உங்களின் சம்மதம் தேவைப்படுகிறது. இந்த தகவல்களை அறிவியல் ஆய்விதழில் வெளியிட உங்களது சம்மதம் எங்களுக்குத் தேவை. நாங்கள் உங்களது குழந்தையின் பெயரையோ அல்லது வேறு அடையாளம் காட்டக்கூடிய தகவல்களையோ வெளியிடுவதை தவிர்ப்போம். மரபியல் பரிசோதனைகளுக்காக உங்களது குழந்தையிடமிருந்து 5 ml இரத்தம் எடுக்க வேண்டி ஏற்படலாம். இந்நோய்க்குக் காரணமான இரண்டு மரபணுக்களிலும் சோதனை செய்த பின்னர், மீதியாக இரத்தம் இருந்தால், அது -80 பாகையில் எதிர்காலத்தில் இந்நோய் சம்பந்தமாக இலங்கையில் நடத்தப்படும் ஆய்வுகளுக்கு பயன்படுத்தப்படும் வகையில் 10 ஆண்டுகள் வரையில் சேமித்து வைக்கப்படும்.

கிடைக்கக்கூடிய நன்மைகள்

இந்த ஆய்வில் நீங்கள் பங்குபெற்றுவதினால், உங்கள் குழந்த்தைக்கு டியுபெரஸ் ஸ்க்லேரோசிஸ் நோய் வர காரணமான மரபியல் குறைபாட்டை அல்லது அறிந்து கொள்ளலாம். இந்த ஆய்வின் மூலம் டியுபெரஸ் ஸ்க்லேரோசிஸ் நோய் பற்றிய அறிவு மேலும் அதிகரிக்கும். அத்தோடு, இலங்கையில் இந்த நோயினால் பாதிக்கப்பட்டோரில் உள்ள நிலைமையை கண்டறிந்து நோயை நிர்ணயிக்க உதவியாக இருக்கும். மேலும், இலங்கையில் இந்த நோயின் நிலைமையை பற்றிய அறிவை வளர்க்க உதவும்.

அபாயங்கள், தீங்குகள் மற்றும் உபாதைகள்.

டியுபெரஸ் ஸ்க்லேரோசிஸ் நோயை உண்டாக்கும் மரபியல் குறைபாட்டை அறிய உதவும் மரபியல் பரிசோதனைகளுக்காக உங்கள் குழந்தையிடமிருந்து 5 ml இரத்தம் எடுக்கப்படும். இந்த செயல்முறை உங்கள் குழந்தைக்கு சிலவேளைகளில் சிறு வலியையோ உபாதையையோ உண்டாக்கலாம். இரத்தம் எடுப்பதால் ஊசி குத்தும் இடத்தில் சிறு சிராய்ப்பு மற்றும் தொற்று ஏற்படக்கூடிய சாத்தியங்கள் உண்டு. பயிற்றுவிக்கபபட்ட ஒருவரால் , கிருமி அற்ற நிலையில் இரத்தம் எடுக்கப்படும் பொது இந்த அபாயங்கள் குறைக்கப்படும்.

செலவு ஈடுகள்

இந்த ஆய்வில் பங்கு பெறுவதற்கு உங்களுக்கோ அல்லது உங்கள் குழந்தைக்கோ எந்தவித செலவும் ஈடுசெய்யப்பட மாட்டாது. இருப்பினும், நீங்கள் மரபியல் சோதனைக்குட்படுத்தப்பட்டால் அதன் முடிவுகளின் நகல் உங்களுக்கு தரப்படும்.

இரகசியத்தன்மை

பெறப்படும் எல்லாத்தகவல்களும் இரகசியமாக பேணப்படுவதோடு, உங்களையோ உங்கள் குழந்தையையோ அடையாளம் காடும் வகையில் ஒருபோதும் வெளியிடப்படமாட்டாது. தகவல் சேகரிப்புக் கையேடு பெறப்படும் தகவல்கள் இரகசியமாகப் பேணப்படுவதை உறுதி செய்யும் வகையில் வடிவமைக்கப்பட்டுள்ளது. தகவல்களைக் கொண்டிருக்கும் இலத்திரனியல் தரவுத்தளம், பங்குபற்றியவர்களின் எண்ணிக்கையை மாத்திரமே கொண்டிருக்கும். மேலும் இத்தகவல்களைக் கொண்டிருக்கும் கணணி கடவுச்சொல் பாதுகாப்பு உள்ளதாக இருக்கும். உங்களது அனுமதி இல்லாமல், தங்களது தகவல்கள் உங்களை அடையாளம் காட்டும் வகையில் பொது மக்களுக்கு முன்னிலைப்படுத்தப்படவோ வெளியிடப்படவோ மாட்டாது.

பங்கு நிறுத்தம்

நீங்கள் உங்கள் குழந்தையை இந்த ஆய்விலிருந்து காரணங்கள் எதுவும் கூறாமல், தண்டங்களோ அல்லது மருத்துவ பராமரிப்பில் பாதிப்புகளோ இல்லாமல் எவ்வேளையிலும் விலக்கிக்கொள்ள முடியும். நீங்கள் விலகிக்கொள்வதாக முடிவெடுத்தால்,தயவு செய்து எங்களுக்கு விரைவில் தெரிவிக்கவும். இருப்பினும், ஆய்வு முடிவுகள் பிரசுரிக்கப்பட்ட பின்னரோ அல்லது பிரசுரிக்க அனுப்பிய பின்னரோ உங்களால் இந்த ஆய்விலிருந்து விலகிக்கொள்ள முடியாது.

விளக்கம்

செய்யப்படும் பரிசோதனைகள்/செயல்முறைகள் பற்றி விளக்கமோ அல்லது தகவல்களோ வேண்டுமெனின் தயவுசெய்து பின்வரும் நபர்களிடம், 011- 2689 545 எனும் தொலைபேசி இலக்கத்தினூடாக தொடர்பு கொண்டு கேட்டறிந்து கொள்ளவும்:

Dr . சமுதிதா சேனாரத்ன

மருத்துவ மரபியல் முதுநிலை கல்வி மாணவி

மனித மரபியல் பிரிவு,

மருத்துவ பீடம்,

கொழும்பு பல்கலைக்கழகம்.

(0777697480, 0113165805)

பேராசிரியர். வஜிர H.W. திசாநாயக,

மருத்துவ மரபியலர்,

மனித மரபியல் பிரிவு,

மருத்துவ பீடம்,

கொழும்பு பல்கலைக்கழகம்.

(0112689545)

CONSENT FORM

A STUDY OF TUBEROUS SCLEROSIS IN A COHORT OF SRI LANKAN PATIENTS

To be completed by the parent/guardian

The parent/ guardian should complete the whole of this sheet himself/herself.

1. Have you read the information sheet? (Please keep a copy for yourself) YES/NO

2. Have you had an opportunity to discuss this study and ask any questions? YES/NO

3. Have you had satisfactory answers to all your questions? YES/NO

4. Have you received enough information about the study? YES/NO

5. Who explained the study to you?
……..

6. Do you understand that you are free to withdraw your child from the study at any time, without having to give a reason and without affecting your child's medical care? YES/NO

7. Sections of your child's medical notes, including those held by the investigators relating to his/her participation in this study may be examined by other research assistants. All personal details will be treated as STRICTLY CONFIDENTIAL. Do you give your permission for these individuals to have access to your child's records? YES/NO

8. Do you agree to have leftover blood samples and DNA is stored for future research into Genetics? YES/NO

9. Do you agree for the samples to be sent abroad? YES/NO

10. Have you had sufficient time to come to your decision? YES/NO

11. Do you agree in your child take part in this study? YES/NO

Parent's/ Guardian's name (BLOCK CAPITALS)

……

Parent's/ Guardian's
signature…….Date……

To be completed by the investigator

I have explained the study to the above parent/guardian and he/ she has indicated his/her willingness in his/her child take part.

Name (BLOCK CAPITALS)

……

Signature of
investigator:……...……...Date……

කැමත්ත ප්‍රකාශ කිරීම

ටියුබරස් ස්ක්ලෙරෝසිස් කොම්ප්ලෙක්ස් සහිත ශ්‍රී ලාංකීය රෝගීන් පිළිබඳ අධ්‍යයනය

මෙම රෝගියාගේ මව /පියා / භාරකරු විසින් සම්පූර්ණ කල යුතුයි.

මව /පියා /භාරකරු විසින් පමණක් සියලු පිළිතුරු සැපයීම කල යුතුයි.

1.ඔබ තොරතුරු පත්‍රිකාව කියවුයේද ? (ඔබගේ පිටපත ලග තබා ගන්නද) (ඔව්/නැත)

2.එහි අඩංගු කරුණු පැහැදිලිව අසා දන ගැනීමට ඔබට අවස්ථාවක් ලැබුනේද? (ඔව්/නැත)

3.ඔබගේ ප්‍රශ්න සඳහා සතුටුදායක පිළිතුරු ලැබුනේද? (ඔව්/නැත)

4.ඔබට මෙම අද්‍යයනය පිළිබඳ සෑහීමකට පත්විය හැකි තරම් අවබෝධයක් (ඔව්/නැත)

ලැබුනේද?

5.මෙම අධ්‍යයනය පිලිබඳව ඔබව දැනුවත් කලේ කවුරුන් විසින්ද? .....

6.අවශ්‍ය ඕනෑම වීටකදී ඔබගේ දරුවා මෙම අධ්‍යයනයෙන් ඉවත්කර ගැනීමට ඔබට නිදහස ඇති බවත් , ඒ සඳහා කරුණු ඉදිරිපත් කිරීම අනවශ්‍ය බවත් , එමගින් ඔබගේ දරුවාට කිසිදු අපහසුතාවයක් සිදු නොවන බවත් , පැහැදිලි වී ඇත. (ඔව්/නැත)

7.ඔබගේ දරුවාගේ වෛද්‍ය වාර්තා අතැම් විට මෙම අධ්‍යයනයට සම්බන්ධ සහයකයින් විසින් අධ්‍යයනය කරනු ඇත.සියලුම පෞද්ගලික වාර්තාවල අතිශය රහසිගතභාවය රකිනු ලැබේ

ඔබේ දරුවාගේ වෛද්‍ය වාර්තා අධ්‍යයනයට අනුමැතිය දෙන්නේද? (ඔව්/නැත)

8.අතිරික්ත රුධිර සාම්පල වෙනත් ජානමය අධ්‍යයනයන් සඳහා යොදාගැනීමට අනුමැතියදෙන්නේද?
. (ඔව්/නැත)

9. රුධිර සාම්පල වෙනත් රටවල දී පරීක්ෂා කිරීමට එකගද ? (ඔව්/නැත)

10. මෙම අධ්‍යයනයට සහභාගී වීම සඳහා තීරණයට එළඹීමට ඔබට අවශ්‍ය පමණ කාල වේලාවක් ලැබුනේද? (ඔව්/නැත)

11. මෙම අධ්‍යයනය සඳහා සහභාගී වීමට ඔබ එකග වන්නේද ? (ඔව්/නැත)

නම (මව /පියා/ භාරකරුගේ) .....

අත්සන.....

දිනය.....

අධ්‍යයනය සිදුකරන නිළධාරීනිය විසින් සම්පූර්ණ කල යුතුය.

අධ්‍යයනය පිලිබඳ සියලු කරුණු පැහැදිලි කරදීමෙන් පසු මෙම රෝගියා ස්වකැමැත්තෙන් ඉදිරිපත් වන ලදී.

නිලධාරිනියගේ නම .....

නිලධාරිනියගේ අත්සන .....

දිනය.....

ஆய்வு அடையாள இலக்கம்: ……

ஒப்புதல் படிவம்

இலங்கையில் டியுபெரஸ் ஸ்க்லேரோசிஸ் (Tuberous Sclerosis) நோயினால் பாதிக்கப்பட்ட நோயாளிகளில் காணப்படும் தோற்றப்பாடுகள் மற்றும் மரபியல் நோய் காரணிகள் பற்றிய பெரும் ஆய்வு.

பெற்றோர்/ பாதுகாவலரினால் நிரப்பப்படவேண்டும்

முழு படிவமும் பங்குபற்றுபவரினால்/ பாதுகாவலரினால் நிரப்பப்பட வேண்டும்.

இந்த ஆய்வை பற்றிய தகவல்களை முழுமையாக வாசித்து தெளிவாக விளங்கிகொண்டீரா? (தயவுசெய்து இந்த தகவல் படிவத்தின் ஒரு பிரதியை நீங்கள் வைத்திருக்கவும்) ஆம்/ இல்லை

இந்த ஆய்வை பற்றி வினாவ உங்களுக்கு சந்தர்ப்பம் கிடைத்ததா? ஆம்/ இல்லை

உங்கள் எல்லாக் கேள்விகளுக்கும் திருப்திகரமான பதில்கள் கிடைத்தனவா? ஆம்/ இல்லை

இந்த ஆய்வைப் பற்றி போதுமான தகவல்கள் கிடைத்ததா? ஆம்/ இல்லை

இந்த ஆய்வைப் பற்றி உங்களுக்கு யார் விளக்கம் அளித்தது?

……

எந்தவித காரணங்களும் தெரிவிக்காமலும் அல்லது உங்கள் குழந்தைக்கு அளிக்கப்படும் மருத்துவ சேவையில் எவ்வித பாதிப்பு ஏற்படாமலும் உங்கள் குழந்தையை இந்த ஆய்விலிருந்து எந்நேரமும் விலகிக்கொள்ள முடியும் என்பதை புரிந்துகொண்டுள்ளீரா? ஆம்/ இல்லை

உங்களது ஆய்வாளர்களிடன் இருக்கும் பதிவுகள் உட்பட உங்களது குழந்தையின் மருத்துவப் பதிவுகள், இந்த ஆய்வில் பங்குபெற்றும் மற்றைய ஆராய்ச்சியாளர்களினால் சிலநேரங்களில் ஆய்வு செய்யப்படலாம். அனைத்து தனிப்பட்ட விவரங்களும் நிச்சயமாக ரகசியமாக பேணப்படும். இந்த ஆய்வாளர்கள் இத்தகவல்களை உபயோகிக்க நீங்கள் அனுமதிக்கின்றீர்களா? ஆம்/ இல்லை

மீதமுள்ள இரத்த மாதிரிகளை எதிர்கால மரபியல் ஆய்வுகளுக்கு பயன்படுத்த அனுமதிகின்றீர்களா? ஆம்/ இல்லை

உங்கள் இரத்த மாதிரியை மேல் பரிசோதனைகளுக்காக வெளிநாடுகளுக்கு அனுப்ப சம்மதிக்கின்றீர்களா? ஆம்/இல்லை

நீங்கள் உங்கள் முடிவுக்கு வர போதுமான நேரம் இருந்ததா? ஆம்/ இல்லை

உங்கள் குழந்தை இந்த ஆய்வில் பங்குபெற்ற சம்மதிக்கின்றீர்களா?

ஆம்/ இல்லை

பங்குபெற்றுபவரின்/ பாதுகாவலரின் கையொப்பம்: ……..

பெயர் (பெரிய எழுத்துக்களில்):

…….திகதி:…….....

ஆய்வாளரினால் நிரப்பப்பட வேண்டியது:

நான், இந்த ஆய்வை பற்றி மேலே குறிப்பிடப்பட்டுள்ள பெற்றோர்/பாதுகாவலருக்கு விளக்கியுள்ளேன். மற்றும் அவர் இந்த ஆய்வில் பங்குபெற்ற சம்மதம் தெரிவித்துள்ளார்.

ஆய்வாளரின்

கையொப்பம்:……திகதி:……..

பெயர் (பெரிய எழுத்துக்களில்):

……...……...

DATA COLLECTION FORM

A STUDY OF TUBEROUS SCLEROSIS IN A COHORT OF SRI LANKEN PATIENTS

Subject Study Number						

Name of
subject……..

Age……

Name of parent
guardian…….

Address…….

……..

……..

Telephone number (Home)……..
(Mobile)……

E mail…….

Referring physician……..

Date of referral……..

Hospital……. Ward……

Clinic No/BHT No…….

Data Protection and Confidentiality

After completion of this page, ensure that the subject study number is entered on **all pages** of this booklet. Then detach this page and store separately from the remainder of the booklet.

Subject Study Number						

Date of entry to study Date on consent form

		-			-				

		-			-				

Date of birth

1. Sex ☐ Male ☐ Female

2. Age of onset

2.1 Features present at birth (i)…….

(ii)……

(iii)…….

2.2 Features appeared after birth (i)……Noticed at……yrs.

(ii)……..Noticed at……yrs.

(iii)……..Noticed at……yrs.

3. Consanguinity ☐ Yes ☐ No

3.1 Family tree

I
II
III
IV
V

Subject Study Number						

3.2 Additional Information

Location in pedigree	Clinical/other info

4.0 Clinical Manifestations

4.1 Skins

Hypomelanotic Macules** ☐ Absent ☐ Present #.....

Fibrous cephalic plaques** ☐ Absent ☐ Present #.....

Ungual Fibromas** ☐ Absent ☐ Present #.....

Shangreen Patch** ☐ Absent ☐ Present #.....

Confetti skin lesions* ☐ Absent ☐ Present #.....

Angiofibromas** Grade: 0 1 2 3

[0 – None, 1 – Macular(Flat)lesions, 2 – Papular lesions < 3mm, 3 – Papular lesions > 3mm and / or extending to below mouth]

4.2 Brain

Seizures ☐ Never ☐ Ever Present ☐ Chronic

Type of Seizures ☐ Infantile spasms ☐ Generalized

☐ Partial ☐ Intractable ☐ Family history

Cortical Tubers** ☐ Absent ☐ Present #.....

Sub Ependymal Nodules** ☐ Absent ☐ Present #.....

Subject Study Number						

Sub Ependymal Giant cell Astrocytomas** ☐ Absent ☐ Present #.....

4.3 Kidney

Renal Angiomyolipomas** Grade: 0 1 2 3

[0 – None, 1 – One or more, all < 1 cm, 2 – Multiple, One or more > 1cm, all < 4cm, 3 – Multiple, One or more > 4cm]

Renal Cysts* Grade: 0 1 2 3 4

[0 – None, 1 – 1 to 2 small (< 2cm), 2 – More than 2 small(< 2cm), 3 – More than 2 and at least one > 2cm, 4 – Classic polycystic kidney disease, multiple cysts with renal enlargement]

4.4 Liver

Hepatic Angiomyolipomas** Grade: 0 1 2 3

[0 – None, 1 – One or more, all < 1 cm, 2 – Multiple, One or more > 1cm, all < 4cm, 3 – Multiple, One or more > 4cm]

4.5 Eye

Retinal Hamartomas** ☐ Absent ☐ Present #.....

Retinal Acromic Patches* ☐ Absent ☐ Present #.....

4.6 Heart

Cardiac Rhabdomyomas** ☐ Ever Present ☐ Never

4.7 Lung

Lymphangiomatosis** ☐ Severe ☐ Mild ☐ Absent

4.8 Mouth and Teeth

Dental Enamel Pits* ☐ Absent ☐ Present #.....

Intra Oral Fibromas* ☐ Absent ☐ Present #.....

** - Major Criteria No…….. * - Minor Criteria No……..

Subject Study Number						

5 Investigation Findings

Investigation	Date	Findings and conclusions
MRI Scan		
CT Scan		
Ultra Sound Scan		
EEG		
2D Echo		
Biopsy Histology		
Other		

6 Behavioral / Neuropsychiatric phenotypes

Mental Retardation Grade: 0 1 2 3

[0 – None (reading above 3rd Grade), 1 – Mild(reading at < 3rd Grade), 2 – Moderate (some speech no reading), 3 – Severe (no speech intelligible to a stranger)]

Subject Study Number						

Autism ☐ Severe ☐ Mild/Moderate ☐ Absent

CARS 2 | | 60 |

[Childhood Autism Rating Scale 2]

7 Genetics

7.1 Pre test Counseling ☐ Done ☐ Not done

7.2 Genetic Testing ☐ Done ☐ Not done

TSC 1 sequencing ☐ Pos ☐ Neg ☐ Not tested

TSC 1 MLPA ☐ Pos ☐ Neg ☐ Not tested

TSC 2 sequencing ☐ Pos ☐ Neg ☐ Not tested

TSC 2 MLPA ☐ Pos ☐ Neg ☐ Not tested

7.3 Mode of Inheritance ☐ *de novo* ☐ Inherited ☐ Not known

7.4 Post test Counseling ☐ Done ☐ Not done

8 Social Status

Education level of parents

Father Grade: 0 1 2 3 4

Mother Grade: 0 1 2 3 4

[0 – Not gone to school, 1 – Schooling up to Grade 5, 2 – Schooling up to Grade 10, 3 – Schooling up to Grade A/L, 4 – Graduate]

Employment ☐ Father ☐ Mother ☐ Both

Monthly income Rs……

Who looks after the child……

Subject Study Number						

Schooling ☐ Yes ☐ No

If schooling ☐ Normal school ☐ Special school

9 Treatments

9.1 Drug Treatments (i)……

(ii)……

(iii)……..

9.2 Surgical Treatments (i)……

(ii)……

(iii)……..

Final Diagnosis……..

Subject Study Number						

Patient Date | | | - | | | - | | | | |

	Label	Volume	Storage	Comments
K/EDTA vial	1. 2.	1. 2.	1. 2.	

COMMENTS

Record reasons for missing data and any additional relevant comments. ENSURE THAT ANONYMITY IS PRESERVED.

The booklet should be signed when ALL available data have been entered and cross checked with relevant data recorded elsewhere in this booklet.

Signed……. Date……

Investigator/Research Assistant

References:

1. Sandra L. Dabora, S.J., David Neal Franz, *Mutational Analysis in a Cohort of 224 Tuberous Sclerosis Patients Indicates Increased Severity of TSC2 Compared with TSC1 Disease in Multiple Organs.* 68 Hum. Genet, 2001: p. 64-80.
2. Northrup, H. and D.A. Krueger, *Tuberous sclerosis complex diagnostic criteria update: recommendations of the 2012 international tuberous sclerosis complex consensus conference.* Pediatr Neurol, 2013. **49**(4): p. 243-54.
3. Krueger, D.A.N., H., *Tuberous sclerosis complex surveillance and management: recommendations of the 2012 international tuberous sclerosis complex consensus conference.* Pediatr Neurol, 2013. **49**(4): p. 255-65.1.

Appendix 4: Genetic testing details & primer sequences

Appendix 4 contains the genetic testing in detail and the primer sequences , probes used in Sanger sequencing and MLPA techniques

PCR procedure in detail

In the first step of the PCR, original double stranded DNA (dsDNA) is denatured applying a high temperature of 95°C so that the hydrogen bond between the two complementary DNA threads are separated into single stranded DNA (ssDNA). The ssDNA works as a template for the target sequence, and when the reaction is cooled down to 60°C, the primers are able to anneal to the ssDNA.

At the next step, DNA polymerase begins synthesizing new DNA strands from the end of the primer; this part of the PCR is called extension and happens at a temperature of 72°C.

See table 1 for the PCR program.

Table 1: PCR Program

PCR M13_360			
Enzyme activation	95°C	10min	
Denaturation	95°C	30sec	
Annealing	60°C	30sec	**30 cycles**
Extension	72°C	1min	
Final extension	72°C	7min	
Soak	10°C	∞	

As PCR cycles through these thermal steps, newly synthesized DNA is used as templates for replication, setting in a chain reaction where the DNA template is exponentially amplified. In this project we have used the AmpliTaq 360DNA polymerase kit form Applied Biosystems.

After PCR the products were purified with Agencourt's AMPure XP beads using a Biomek FXP automated system (Beckman Coulter). The AMPure XP beads utilize Agencourt's solid-phase paramagnetic bead technology and removes excess primers, nucleotides, salts and enzymes. (https://www.beckmancoulter.com).

Primer design

The primers are 20-25 bp long and have a M13 tail at the 5'end. The forward primer is tailed with the M13 F sequence 5'- tgtaaaacgacggccagt-3' and the reverse primer is tailed with M13 R sequence 5'- caggaaacagctatgacc-3'. This tailing makes it possible to use the same complementary primers for sequencing the target sequence after PCR. The PCR primers are also designed with the same melting temperatures, which allow a better workflow as one can use the same PCR program for all the reactions. Table 2 and 3 shows the primer sequence in the upper case, and the M13 tailing in lower case for *TSC1* and *TSC2* respectively.

Table 2: Primer sequence of coding regions in *TSC1*

Exon	Primer pair	Primer sequence ('5->'3)	Design
3	TSC1_3F	tgtaaaacgacggccagtTGCATGATTCTTACTTCATGTGTGTGC	RSA000572713
3	TSC1_3R	caggaaacagctatgaccCCCTCTTCATAAACTCGCCAAAGA	RSA000320209
4	TSC1_4F	tgtaaaacgacggccagtGAACAATGTCATCAGTGGCGCA	RSA000320209
4	TSC1_4R	caggaaacagctatgaccGGCAGAACTGTAATGCTGCACAA	RSA000031280
5	TSC1_5F_v2	tgtaaaacgacggccagtTTGAGAAAAGCCAAATGCCTA	Primer 3
5	TSC1_5R	caggaaacagctatgaccTCAGGCCAAATGCAGCAGTG	RSA000320211
6	TSC1_6F	tgtaaaacgacggccagtTGGATGCACCCAAGATATTCCC	RSA000320211
6	TSC1_6R	caggaaacagctatgaccGAACTCGTCTCCCGGATGCC	RSA000932011
7	TSC1_7F	tgtaaaacgacggccagtTTTCCCTGTCTGCCGTTAAATACAA	RSA000932011
7	TSC1_7R	caggaaacagctatgaccAAGGGTGTCTGGTGTGTTCGGA	RSA000572708
8	TSC1_8F	tgtaaaacgacggccagtGGACAGGCACTTGTGCTGCAA	RSA000572708
8	TSC1_8R	caggaaacagctatgaccTCCTCGCCACTCCAATCCCT	RSA000031246
9	TSC1_9F	tgtaaaacgacggccagtCACGTCACCAACTCTGTTGGCA	RSA000031246
9	TSC1_9R	caggaaacagctatgaccGGCATGGTCCCGCTTGTTCT	Primer 3
10	TSC1_10F	tgtaaaacgacggccagtCATACTAAATCTGACCCAAAG	Primer 3
10	TSC1_10R	caggaaacagctatgaccTAAAACCACACACTAACCC	RSA000031244
11	TSC1_11F	tgtaaaacgacggccagtCCCAGGGATTTGCAATAAGTGTCA	RSA000031244
11	TSC1_11R	caggaaacagctatgaccTGTGCCTGCTCTCTCCTCTGC	RSA000572696
12	TSC1_12F	tgtaaaacgacggccagtGGGTCGGCAGATCACACCTTG	RSA000572696
12	TSC1_12R	caggaaacagctatgaccTTTGACACTTATTGCAAATCCCTGG	RSA000064859
13	TSC1_13F	tgtaaaacgacggccagtTCTTCATGCTGAACAGAGAAGGC	RSA000064859
13	TSC1_13R	caggaaacagctatgaccCCCAACAATTTGAGAATCACTGCAC	RSA001290665
14	TSC1_14F	tgtaaaacgacggccagtTGGCATCACTTTACCTGGCATAGG	RSA001290665
14	TSC1_14R	caggaaacagctatgaccGGACAGAGCCATGTCCAGCC	RSA000031237
15	TSC1_15aF	tgtaaaacgacggccagtAAGCCCACTCTCGTCGGAGG	RSA000031237
15	TSC1_15aR	caggaaacagctatgaccGACACTGGCATGTGGCAGCA	RSA000031223
15	TSC1_15bF	tgtaaaacgacggccagtGCAGAGAACCAGCTGCCTCAA	RSA000031223
15	TSC1_15bR	caggaaacagctatgaccTCGGCAGCCTCCAGTTCTCA	RSA000031221
16	TSC1_16F	tgtaaaacgacggccagtCAGGGAAGCCTGGCAGGAAG	RSA000031221
16	TSC1_16R	caggaaacagctatgaccCCTGCAACCCTCTCCGCTCT	RSA000031219
17	TSC1_17F	tgtaaaacgacggccagtTGCCCAAAGGAGTGGGAAGG	RSA000031219
17	TSC1_17R	caggaaacagctatgaccCAGGGCATCTGAAATGCGTGA	RSA000320227
18	TSC1_18F_v2	tgtaaaacgacggccagtGCTGAACAAGTCAAGGACACC	Primer 3
18	TSC1_18R	caggaaacagctatgaccTGGAAGCAAACTGATCCCTGAGA	RSA000031217

19	TSC1_19F	tgtaaaacgacggccagtCCATGGGATACACATAGAGCAGGG	RSA000031217
19	TSC1_19R	caggaaacagctatgaccGGGCCCAAGAAAGTAGAGCCG	RSA000320229
20	TSC1_20F	tgtaaaacgacggccagtTAGTGGGACTGCCGCTCCGT	RSA000320229
20	TSC1_20R	caggaaacagctatgaccTATGCCACTGGGCCTTGGTG	RSA001303487
21	TSC1_21F	tgtaaaacgacggccagtGCCATCTTTCTCCAACCTGCC	RSA001303487
21	TSC1_21R	caggaaacagctatgaccTTTATGTTTCACATGATTTGCTTGGG	Primer 3
22	TSC1_22F	tgtaaaacgacggccagtCGGAGTGAGCTGAGTGTTGCAG	RSA000572684
22	TSC1_22R_v2	caggaaacagctatgaccAAACGGATTTTGGAACTGGAATC	Primer 3
23	TSC1_23F_v2	tgtaaaacgacggccagtTGTCAGTGTAATTCCACATCCTC	Primer 3
23	TSC1_23R	caggaaacagctatgaccCAGACCCTGGAAACAGGAAA	RSA000023560

Table 3: Primer sequence of coding regions in *TSC2*

Exon	Primer pair	Primer sequence ('5->'3)	Design
1	TSC2_1F	tgtaaaacgacggccagtCTGGTGTCTCCCGGGCTTTC	RSA000023560
1	TSC2_1R	caggaaacagctatgaccGGAAGGACATGTCGGCCTCC	RSA000320763
2	TSC2_2F	tgtaaaacgacggccagtTCGTCAAGTGAATCTTGATTCCAGAAA	RSA000320763
2	TSC2_2R	caggaaacagctatgaccGTGCAAACCAGATCATCGGCA	RSA000023545
3	TSC2_3F	tgtaaaacgacggccagtTCCAGGTGAGGCCACCATTG	RSA000023545
3	TSC2_3R	caggaaacagctatgaccTCCAGCTCCAGAGCCCATCA	RSA000320759
4	TSC2_4F	tgtaaaacgacggccagtCGGGCAGGAGCTGTGTCATC	RSA000320759
4	TSC2_4R	caggaaacagctatgaccAGGTGAGCCCAGGTGCATGA	RSA001284317
5	TSC2_5F	tgtaaaacgacggccagtGTCTGTCTGTTGCTGCCGGG	RSA001284317
5	TSC2_5R	caggaaacagctatgaccTGGTGGTTTCAACTTTATTCACTGCG	RSA000321770
6	TSC2_6-7F	tgtaaaacgacggccagtAGAGTGACTAGACCACAGCCCGT	RSA000321770
6	TSC2_6-7R	caggaaacagctatgaccACGATGAACAGCGGGAGGCT	RSA001301781
7	TSC2_7F	tgtaaaacgacggccagtTGACCCACAGTGACAGGGACG	RSA001301781
7	TSC2_7R	caggaaacagctatgaccAGCTCGCCACCATCTCCTCC	RSA000321769
8	TSC2_7-8F	tgtaaaacgacggccagtAGCCTCCCGCTGTTCATCGT	RSA000321769
8	TSC2_7-8R	caggaaacagctatgaccTGCTTGGCAAGGGACACTGG	RSA000320755
9	TSC2_9F	tgtaaaacgacggccagtGCCTGTGCGCAGGAGTGAAC	RSA000320755
9	TSC2_9R	caggaaacagctatgaccCCAGCTGCAAAGCAACTGCC	RSA000320754
10	TSC2_10F	tgtaaaacgacggccagtCGGGTGCCCAGGATTCAGTT	Primer 3
10	TSC2_10R	caggaaacagctatgaccGATGAGCGCCACTGCGAATC	RSA000320753
11	TSC2_11F_v2	tgtaaaacgacggccagtGGGGGTGTCTCAACCCATGA	RSA000320753
11	TSC2_11R	caggaaacagctatgaccTTCCCTGCCGAGTGCAGAAA	RSA000023503
12	TSC2_12F	tgtaaaacgacggccagtTTTGTGTCTGGGCTGTGGGC	RSA000023503
12	TSC2_12R	caggaaacagctatgaccGCCCAGTGGCGTCATGAGAG	RSA000023502
13	TSC2_13F	tgtaaaacgacggccagtTCTCATGACGCCACTGGGCT	RSA000023502
13	TSC2_13R	caggaaacagctatgaccCGTGTCAAGGCCTGGCAGAG	Primer 3
14	TSC2_14F	tgtaaaacgacggccagtCCAGCTGTGCTGAAGTCCCG	RSA000574139
14	TSC2_14R_v2	caggaaacagctatgaccCAGGACACAAATCCAGCAGTGG	RSA000946792
15	TSC2_15F_v2	tgtaaaacgacggccagtGGTGTTTGTGGTAGAAAGTGTTCTCA	Primer 3
15	TSC2_15R	caggaaacagctatgaccATCCAGGGAGGGTGTGGGTC	RSA000321666
16	TSC2_16F	tgtaaaacgacggccagtATCGCCCTGCAGCACACACT	RSA000321666
16	TSC2_16R	caggaaacagctatgaccGCAGAGGAGGGACGGTCAGC	Primer 3
17	TSC2_17-18F	tgtaaaacgacggccagtGCACATCAGCAGGTGGCCTT	RSA000574149
17	TSC2_17-18R_v2	caggaaacagctatgaccAGCAGGCAGGTGGCAGCTC	Primer 3
19	TSC2_19F	tgtaaaacgacggccagtTCCCTCCCTGTCTGGCCTGT	RSA001322468
19	TSC2_19R_v2	caggaaacagctatgaccTGCAGCTGAGTTTGAGGGAAA	RSA001322469
20	TSC2_20F	tgtaaaacgacggccagtTCCTGGGAGGGAGGCAAGAA	RSA001322469
20	TSC2_20R	caggaaacagctatgaccCCCAGGGAAGCAGAGCCAAC	Primer 3
21	TSC2_21F	tgtaaaacgacggccagtCCTGTGGGATCGTGTCGGAA	RSA000023474

21	TSC2_21R_v2	caggaaacagctatgaccCAGAGAAGCCCGCACAGGTT	RSA000574153
22-23	TSC2_22-23F	tgtaaaacgacggccagtGTGTGGCCGTGGCCTTCTCT	RSA000574153
22-23	TSC2_22-23R	caggaaacagctatgaccACTGCCCTCCACCTGCCTGT	Primer 3
24	TSC2_24F	tgtaaaacgacggccagtACAGGCAGGTGGAGGGCAGT	RSA001300396
24	TSC2_24R_v2	caggaaacagctatgaccTGAGGGGCTGAGGGGTGTTA	RSA000023467
25	TSC2_25F	tgtaaaacgacggccagtACTGCCCTTTGGCATGGCTC	RSA000023467
25	TSC2_25R	caggaaacagctatgaccGCAACCAGCCCGTGCTCATA	RSA000574166
26-27	TSC2_26-27F	tgtaaaacgacggccagtAAGCTGAGGCTCGCTGGGC	RSA000574166
26-27	TSC2_26-27R	caggaaacagctatgaccGACAGCCGGTGTCGAGGCT	Primer 3
27-28	TSC2_27-28F_v2	tgtaaaacgacggccagtAAGCTGAGGCTCGCTGGGC	Primer 3
27-28	TSC2_27-28R_v2	caggaaacagctatgaccTGGGACCAGCAGCAACTGAG	RSA000023460
29	TSC2_29F	tgtaaaacgacggccagtAGCCCTTCTCTGCTCCAGCG	RSA000023460
29	TSC2_29R	caggaaacagctatgaccCCCAAGAGGGCCAAGTCTGC	RSA000320733
30	TSC2_30F	tgtaaaacgacggccagtTCCGGACTGTGAGGCTGTGG	RSA000320733
30	TSC2_30R	caggaaacagctatgaccACAATGGTGCTGAGGCAGGC	Primer 3
31	TSC2_31F_v2	tgtaaaacgacggccagtCTGTGGCTGCAGATGGCACT	Primer 3
31	TSC2_31R_v2	caggaaacagctatgaccACAGGGCTCAGCCACAAAGG	Primer 3
32	TSC2_32F	tgtaaaacgacggccagtACTAGGGTGGGCAGAGCCGA	RSA000320731
32	TSC2_32R_v2	caggaaacagctatgaccTGCACGAGGGATGTGGAAGA	RSA000574171
33	TSC2_33aF	tgtaaaacgacggccagtGTGCTCGGGCTGGTCTGTG	RSA000574171
33	TSC2_33aR	caggaaacagctatgaccCCTGGCACTTTCTCTGCATTGG	RSA001284361
33-34	TSC2_33b-34F	tgtaaaacgacggccagtCCCTGAGGTTAAGGCCCGGT	RSA001284361
33-34	TSC2_33b-34R	caggaaacagctatgaccGATGCAGGAGAGGGAGGCCA	RSA000320727
34-35	TSC2_34-35F	tgtaaaacgacggccagtCTCACCTGGGTGCCCACCAT	RSA000320727
34-35	TSC2_34-35R	caggaaacagctatgaccAACCTGTCACTCGCACCGGG	RSA000025404
36	TSC2_36F	tgtaaaacgacggccagtTGACACCCGATGTCTGGCCT	RSA000025404
36	TSC2_36R	caggaaacagctatgaccCAGTGGTCCTCGGCTCTCCC	RSA000023438
37	TSC2_37F	tgtaaaacgacggccagtTAACAGCGTGGGCATGGAGG	RSA000023438
37	TSC2_37R	caggaaacagctatgaccGAGGACACAACGCAGGCTCG	RSA000321417
38-39	TSC2_38-39F	tgtaaaacgacggccagtGGTGTCTTGCCTGTGGCTGC	RSA000321417
38-39	TSC2_38-39R	caggaaacagctatgaccATTTGCGTGCAGGGCCATCT	Primer 3
39-40	TSC2_39-40F	tgtaaaacgacggccagtACGTGATCGTCACCCCGCTG	Primer 3
39-40	TSC2_39-40R	caggaaacagctatgaccCTGGGGTTGGAGTAGGCGGC	Primer 3
41	TSC2_41F	tgtaaaacgacggccagtAATATGGGGCTCCCTCAGC	Primer 3
41	TSC2_41R	caggaaacagctatgaccGACAGGCAATACCGTCCAAG	Primer 3

Sanger sequencing procedure in detail

For sequencing, a mixture of target DNA template, short DNA primer complementary to target DNA ,DNA polymerase, 4 nucleotides and 4 dideoxynucleotides with fluorescent tags are used.

The process starts with heating the mixture 96°C until the two complementary DNA strands separate. Then the temperature lowers to 50°C and the primers bind to the template. In the next step the temperature is raised up to 60°C and the enzyme binds to DNA to create a copy of the template DNA. Detailed sequencing program is presented in Table 4.

Table 4: Sequencing program

Sequence			
Denaturation	**96°C**	**10sec**	
Annealing	**60°C**	**5sec**	**25cycles**
Extension	**50°C**	**4min**	
Soak	**10°C**	∞	

The sequence of newly created copy of the template DNA is complementary to the original DNA template. DNA polymerase incorporates both nucleotides and fluorescently tagged ddNTDs. When the ddNTDs is incorporated, the reaction is terminated. The ddNTDs can be incorporated at any position in the strands, and the strands will be terminated at different positions resulting in a mixture of strands with many different lengths. Once the sequencing reaction is completed, the mixture is purified using Agencourt's CleanSEQ® magnetic beads and the unwanted material is removed with mainly the same principle as Agencourt's AMPure beads (https://www.beckmancoulter.com).

Multiplex Ligation dependent Probe Amplification procedure in detail

At the first step DNA was denatured and incubated for 20 hours with a mixture of MLPA probes for hybridization. We used the SALSA MLPA probekit P124-C1- 0112 TSC1 and the SALSA MLPA probekit P046-C1- 1011 TSC2, both products from MRC-Holland, and consisting of minimum one probe pair per exon (www.mlpa.com). A thermal cycler was set up to run MLPA program with a denaturation temperature of 98°C, then cooling down to 25°C, again denaturation at 95°C and hybridization at 60°C. During this overnight process, the probes are hybridized to immediately adjacent target sequences. The ligation reaction was started by adding the ligation mix to each sample.

The ligation program was run at 54°C for 15 minutes. Only the probes that were hybridized to immediately adjacent target sequences could be ligated during this ligation reaction. After the ligation reaction, we used PCR to amplify the number of probes. It was performed using Veriti 96 well thermal cycler by Applied Biosystems with MLPA – PCR program. See Table 5 for details on the program.

Table 5: MLPA PCR program

MLPA PCR program			
Denaturation	**95°C**	**30sec**	
Annealing	**60°C**	**30sec**	
Extension	**72°C**	**1min**	**35 cycles**
Final extension	**72°C**	**20min**	
Soak	**15°C**	**∞**	

Ligated probes were amplified with this PCR. The number of probe ligation products is a measure of the number of target sequences in the sample. The amplification products were separated using capillary electrophoresis. The amplified products which were separated generate a signal, and these results were interpreted using Gene Marker® software program. (For full probe sequence go to www.mlpa.com).

Appendix 5: Classification of sequence variants

Vedlegg til:
Sekvensvarianter - Vurdering

Classification of sequense variants at Dept. of Med. Gen., Ullevål

Pathogenic = destructive for protein production and/or function

Well documented normal variant

Not well documented, but likely to be normal variant

Not documented uncertain variant (VUS – variant of unknown significance)

Not documented, but likely to be pathogenic variant

Well documented pathogenic variant

Documented neutral variants:

Reports in databases with ”normal controls” with data on frequency.

Available databases:

DbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/)

NHLBI exome sequencing project (http://evs.gs.washington.edu)

1000 genomes (http://browser.1000genomes.org)

Frequency must be considered in context of

population size

relevand disease (penetrance, ekspressivity, age of onse)

mode of inheritance (dominant, recessive)

Papers reporting variants in normal controls.

Seen in our own patient material.

Found in ”in house” controls.

Recurrent mutations in class 5.

When X-linked inheritance with homogeneous expressivity and complete penetrance is found in healthy father, it can be concluded that the variant is neutral.

Not well documented, but likely to be normal variant:

Synonymous variants that are likely to be neutral, but lack sufficient documentation in order to belong to group 1.

Exeption from rule:

The variant changes one of the last three or three first bases of an exon

The variant introduces a new splice site (tested in prediction tools in Alamut)

Intron variants outside consensus splice site, which are probably neutral variants, but lack sufficient documentation in order to belong to group 1.

If prediction tools predicts that a de novo splice site has been made, nest step could be to analyse mRNA, given that information exists on where the protein is expressed..

Missene variants that are reported to be neutral (e.g. do not segregate with disease).

Class 1 and 2 will not be sent out in report, only in rare occasions where the disorder has 100% penetranc and when the mutation is de novo.

Variants of uncertain significance (and lack of documentation):

Usually missense and splice site variants (without documentation), however, separate considerations might move them to class 2 or 4.

Missense:

Generally:

Pathogenicity clues from AlaMut is inkluded in VUS evaluation form, but value of these clues is very uncertain.

Conservation between species. NCBI HomoloGene

Individual evaluation according to protein:

Does the amino acid change involve:

Cysteins involved in S-S-bindings?

Binding sites for co factors, such as Ca^{2+} ?

An AA that is known to be modified (glycosylated, phosphorylated etc)?

An AA which is involved in a binding site for other proteins?

(UniProt/litteratur)

Is the variant located in a well characterised and important domene?

(UniProt, litteratur)

Are there known /reported other pathogenic variants in same location?

Are there known /reported other pathogenic variants close to the location?

(HGMD, PubMed, Google)

Variant in consensus splice site:

Consensus splice site [$(n)_{20}$XXX----------XXXnnnnnn]:

donor splice site: 3 last bases of exon (X) and 6 first bases of intron (n)

acceptor splice site: 20 last bases of intron (n) and first bases in exon (X)

Prediction tools (AlaMut): MaxEntScan

NNSPLICE

GeneSplicer

Human Splicing Finder

4.Not documented, but likely to be pathogenic variant;

Nonsense og frame shift variants

Premature stop codon (PSC) is likely to activate nonsense mediated mRNA decay (NMD.

NB: NMD will only be activated if PSC is appr. 50 bp upstream om last exon-intron boundary. If PSC is located in a C-terminal tail of the protein, it can lead to a truncated, but functional protein.

Splice site

Variants in +1,+2----- -2,-1 can be regarded as pathogenic (very few exeptions).

Other issues

De novo variants in genes with good association to phenotype can be regardes as pathogenic.

Class 3 and 4 variants are to be reported. If a class 3 variant is identified in addition to a variant from class 4 or 5, it may be omitted from the report.

Consider to check for cosegregation /familystudies, or mRNA analyses.

Well documented pathogenic variants

When previously identified and evaluated in the department and/or fulfills the following:

Well documented in publications:

Previously identified in at least 2 patients, and described in 2 independent papers

De novo inheritance in patient

Well described in >1 publication

Well described in 1 publication, and supported by other studies (family studies or functional studies)

Reported in a locus specific database and/or in HGMD database alone, is not sufficient. Published documentation is necessary.

If the variant is ever identified in healthy controls, the frequency has to be according to knowledge on mode of inheritance, penetrance and expressivity for the relevant disease.