Clinical and Molecular Genetics of Tuberous Sclerosis Complex

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Clinical and molecular genetics of tuberous sclerosis complex

Klinische en moleculaire genetica van tubereuze sclerosis complex

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Opgedragen aan Yvonne,
Eveline, Caroline en Rogier

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CHAPTER 1

INTRODUCTION

1.1
Historical Perspective

The hereditary disorder tuberous sclerosis or tuberous sclerosis complex (TSC) is a multi-system disorder with primarily neurological and dermatological signs and symptoms. The history of the original reports about the clinical picture of TSC was well described by e.g. Donegani et al. (1972) and by Gomez (Tuberous sclerosis 1979). Manuel R. Gomez, paediatric neurologist at the Mayo Clinic (Rochester, Minnesota), is considered the 'godfather of TSC research' in the last decades. His monograph on TSC, the third edition of which appeared in 1999, has made an immense contribution to the basic knowledge, awareness and understanding of both scientists and clinicians about the very diverse clinical spectrum of this truly complex disorder.

1.1.1 Literature on TSC

When reviewing the medical literature on TSC, three historical periods can be distinguished. The first period is taken from about the 1850's until the end of the 19th century. This was the time of clinical descriptions of most hamartomatous syndromes, published mainly in case reports. Diseases like Von Hippel Lindau disease, Von Recklinghausen's neurofibromatosis (neurofibromatosis type 1) and TSC (Morbus Bourneville-Pringle) were described. Often the case descriptions were eloquently written and were descriptive rather than analytical. The association between the symptoms of the skin and those of the central nervous system became recognised and was explained in the light of the common ectodermal origin of both these tissues. At a later stage the term phakomatosis was proposed to cover the group of diseases of (neuro)ectodermal origin (van der Hoeve 1920).

Von Recklinghausen (1862), whose name is primarily associated with neurofibromatosis type 1, was probably the first person to publish a case report about a TSC patient. He described multiple myomas in the heart from a newborn baby that died immediately after birth. In the brain of the child a considerable number of 'scleroses' (Sklerosen) were found. This case report was written at a time that TSC was a descriptive pathological diagnosis rather than a well-known clinical spectrum of disease.

Désiré-Magloire Bourneville (1880) published clinical details of a severely retarded 12-year-old girl, with skin signs, that would now be described as facial angiofibroma and molluscum fibrosum pendulum. She probably represented a new mutation, as her parents were unaffected. She was not able to speak or walk, had a hemiplegia and showed severe epilepsy, sometimes with more than fifty seizures per day. Autopsy performed after her death at the age of twelve years showed cortical tubers and tumours in the corpus striatum in her brain. Also her kidneys showed multiple tumours.

Several years later, Balzer and Ménétrier (1885) presented a case report of adenoma sebaceum: a patient with adenoma sebaceum of face and scalp, with some of the lesions located on the upper lip. This latter localisation would later turn out to be rather unusual. They

gave the name 'adenoma sebaceum, tubulous variety' to the condition because of a preponderance of sebaceous gland tissue as observed under the microscope, and in order to distinguish these lesions from epitheliomas.
Pringle (1890) was the first author to write about adenoma sebaceum in the English literature. He described a girl with sebaceous adenoma with 'intelligence [...] decidedly below par', with small, carious teeth, and no lesions on upper lip or scalp. He included a further two cases of adenoma sebaceum communicated to him by colleagues, and referred to the Balzer and Ménétrier publication five years earlier. In line with their findings, Pringle's conclusion was that hypertrophy of the sebaceous glands was the essential element in these lesions, and that the condition was either congenital or observed in early life and that it was often aggravated at the time of puberty. Other features he described, were frequent recurrence after surgical removal, concomitant vascular hypertrophy or telangiectasias, and the common presence of abnormal pigmentation of the skin. Caspary (1891) described adenoma sebaceum in a 17 year old girl, recognised as such following Pringle's publication, and discussed a differential diagnosis with acné, milia, molluscum contagiosum and xanthomas. Caspary's patient was reported to be of normal intelligence and probably was a sporadic patient (due to a new mutation), since she was the first and only person in her family with adenoma sebaceum.
Taylor and Barendt (1893) reported multiple cases in a single family. The index patient had adenoma sebaceum and had been seen by Taylor two years earlier, after he had read the publication by Pringle. The patient's father and two brothers, one brother with epilepsy and another brother described as 'idiot', had shown similar eruptions of adenoma sebaceum. Taylor and Barendt made the observation that adenoma sebaceum is especially prevalent in the less intelligent and that 'psychic disturbance was almost invariably present'. They also described a disagreeable odour coming from the secretions from the 'vesicles', and agreed with the hypothesis that the skin condition originated from sebaceous gland tissue.

The second period dates from the beginning of this century until 1979. In this period more and more case reports of tuberous sclerosis complex patients appeared, gradually providing a complete clinical picture of TSC. Vogt (1908) published his famous triad of 'epilepsy, mental retardation and adenoma sebaceum', defining the core symptoms of the TSC spectrum for decades to come. Later the term 'epiloia' came into use in the English literature, originating from the combination of 'epilepsy' and 'anoia' (= minded-less), but later also used as an eponym for epilepsy, low intelligence and adenoma sebaceum (Vogts triad).
The autosomal dominant pattern of inheritance of TSC, and the high proportion of seemingly sporadic cases, became gradually recognised. Adamson (1911) described a mother and son with adenoma sebaceum, seemingly congenital in the mother, and diagnosed at five years of age in the son. Both patients were mentally normal. Berg (1913) published a short report on adenoma sebaceum in a patient, who had died from a renal tumour at 28 years of age. The patient had adenoma sebaceum from the age of 4 years, and his first epileptic seizure had occurred at age 20. At autopsy, 'tuberous sclerosis' of his brain was diagnosed. His father had

died from a renal tumour at 64 years of age. The daughter of the patient was clearly affected with TSC, with epilepsy, mental retardation and adenoma sebaceum.

The 1920's were an important decade in TSC research in the Netherlands. The Dutch ophthalmologist van der Hoeve (1920) described retinal lesions in a TSC patient and called them 'phakoma'. Carol (1921), a lecturer in dermatology in Amsterdam, wrote an article drawing attention to the fact that 'adenoma sebaceum' is a misnomer. He described giant cells and a low number of sebaceous glands in the skin of TSC patients, and proposed the alternative term hamartoma pilo-sebaceum, referring to the 'naevoid nature' of the lesions. In 1922, two theses were presented to the Dutch scientific community. In Leiden, Broers completed a dissertation with the (translated) title 'About adenoma sebaceum and tuberous sclerosis accompanying skin abnormalities' (Broers 1922). Broers covered the available literature on adenoma sebaceum and gave a detailed case report of a girl with adenoma sebaceum, who had presented with an eye tumour, had other skin manifestations of TSC and was mentally retarded. In his introduction, Broers mentioned the prevailing theories about the origin and nature of the brain abnormalities in TSC by Pellizi, Geitlin, Alzheimer and Vogt. These theories are worth mentioning in view of what we presently know about the pathogenesis of TSC. Pellizi explained the tuberous sclerosis complex of the brain as a lack of development of specific elements of the brain in the last phases of cortical development. Geitlin proposed the mechanism of impaired development of the neuroblasts or precursors of neuroblasts and subsequent failure to migrate into the cortex, thus giving rise to parenchymal and ventricular foci. Alzheimer's opinion was that TSC in the brain originated from a lack of differentiation of the neuroblasts and spongioblasts. Finally, Vogt saw the cortical nodules, heterotopias and ventricular nodules as a 'tumourous' process and proposed that the giant cells were derived from the primary neural epithelium, before the differentiation into spongioblasts and neuroblasts had occurred (Broers 1922). Current opinions about the origin of the brain abnormalities in TSC have not diverted a lot from the ideas put forward over 70 years ago, and still largely follow the hypothesis, that TSC results from a disturbance in differentiation and migration of the primitive neuro-ectodermal cells.

Also in 1922, in Utrecht van Bouwdijk Bastiaanse published a dissertation about TSC, with the (translated) title: 'A familial form of tuberous sclerosis, clinical and histo-pathological study' (Van Bouwdijk Bastiaanse 1922). This thesis drew attention to the hereditary aspect of TSC and contained an extended discussion on the histopathology of the brain in TSC. Some theories about the aetiology of TSC were covered, roughly grouped as 'infection', 'neoplasm' and 'disruption in development' theories. The conclusion of the thesis was that (translated): 'the abnormalities in brain, kidneys, skin and heart can be explained by [...] a developmental disorder of the parenchyma, with secondary reactive growth of the surrounding tissues'. Thus, the conclusions as presented by Broers were supported by van Bouwdijk Bastiaanse. The author stated that transition from developmental disorder into malignant tumour is rare.

Zappert (1926) described concordant twin brothers with TSC, whose sister had died from heart failure at the age of 11 years and whose father was also affected. A fourth child in this

family had suffered from epilepsy and was considered a patient with early-onset TSC. In his article, Zappert mentioned other familial cases he knew from the literature.
In the thirties, the field of TSC research did not escape eugenic thinking. Delmond and Schwarzman (1939) described clinical variation in a three-generation family, presenting a pedigree illustrating (in their view) a connection between TSC, alcoholism, and aggressive and criminal behaviour. The authors stated that problematic situations arose especially when a TSC family member married into a family with problems of aggressive behaviour and alcoholism. In order to prevent damage and violence, they proposed to consider 'eugenic sterilisation'.
In summary, most literature on TSC in this second period was published in journals with a highly specialised dermatological, ophthalmological or neurological profile, with focus on organ specific pathology and only limited information on the multi-system nature of TSC.

The third period started in 1979, when Dr Manuel R. Gomez, paediatric neurologist at the Mayo Clinic published the first edition of his monograph 'Tuberous Sclerosis'. Gomez was the first person to provide a comprehensive review on TSC, describing the full clinical and subclinical spectrum of the disease. Twelve years earlier, Gomez had been co-author of a prelude to this book (Lagos and Gomez 1967). His monograph contained both published and unpublished data on TSC, and helped to revive the interest of clinicians and a growing number of genetic researchers in this fascinating disorder. In the eighties, families with seemingly incomplete penetrance were reported by geneticists and paediatricians. In the Netherlands, the paediatric neurologist Dr Paul Fleury gave an important stimulus for research into TSC. He meticulously documented families with TSC (Fleury et al. 1980), played an important role in establishing a standard protocol for clinical examination of TSC patients and their relatives (van Baal, Fleury, and Brummelkamp 1990), and helped to refine the diagnostic criteria (Fleury 1989). From his original group of TSC families, several were included in the molecular studies, e.g. Sampson et al. (1989), that have led to the identification of both TSC genes in 1993 and 1997. Genetic research became more prominent with the development of molecular genetic and biostatistical techniques like Southern blotting and linkage analysis. Important hallmarks were the publication of linkage of a possible TSC gene to the ABO blood group locus on chromosome 9q (Northrup et al. 1987), and the prediction of the second locus on chromosome 16 (Kandt et al. 1992). Attempts to distinguish the chromosome 9-linked and not 9-linked families clinically were unsuccessful.
Since then a fourth period commenced. Two genes for TSC were identified, fully sequenced and partly characterised: the TSC2 gene on chromosome 16 (The European Chromosome 16 Tuberous Sclerosis Consortium 1993), and the TSC1 gene on chromosome 9 (van Slegtenhorst et al. 1997). An increasing number of mutations are being reported, and new attempts are being made to evaluate possible genotype-phenotype correlations. In the field of protein biology, the functions of the two gene products, tuberin (TSC2) and hamartin (TSC1), are under investigation.

1.1.2 Aim of this thesis

As a continuation of the family studies by Fleury (1980), the TSC research group at the Erasmus University Rotterdam was formed with the aim of identification of the genetic basis of this disorder and gain more insight in its aetiology. Accurate and standardised phenotypic characterisation of patients and relatives was considered an important tool for a reliable classification of patients and to differentiate TSC from seemingly similar conditions. Subsequently, after the discovery of the genetic basis for TSC, this clinical information would be indispensable for the analysis of a possible relation between genotype and phenotype. Furthermore, knowledge about the genes and mutations involved in TSC would enlarge the possibilities of diagnosis of TSC in patients with partial expression of the disease, relatives of patients, and during pregnancy, and be of significant influence and support for genetic counseling.

In this thesis, the clinical spectrum of TSC, but also conditions that resemble TSC and may confound the diagnosis, are analysed. Secondly, the relation between genotype and phenotype is studied. Thirdly, the clinical genetic application of the newly acquired diagnostic tools is discussed

1.2
Current state of the art

1.2.1 Introduction

Tuberous sclerosis complex (TSC) is a hereditary disorder belonging to the group of neuro-ectodermal disorders, that have in common a combination of neurological abnormalities (epilepsy, mental retardation, structural brain abnormalities), abnormalities of the skin (disorders in pigmentation), and benign tumour growths: hamartomas. Besides TSC (Morbus Bourneville Pringle), the most prevalent diseases in this quite heterogeneous group are M. Von Recklinghausen (neurofibromatosis type 1), Von Hippel Lindau disease and Sturge-Weber angiomatosis. However, it was recognised early that the symptoms of these conditions not only involve the derivatives of the outer ectodermal germinal layer, but may affect mesodermally derived tissues as well.

The credit of the first accurate description of the brain pathology of the TSC is generally given to Bourneville, who proposed the name "sclérose tubéreuse" for the disease (Bourneville 1880). Earlier, in 1863, congenital tumours in the heart and 'scleroses' in the brain of a new-born child had been described (von Recklinghausen 1862). Recklinghausen's case report is considered the first clinical description of a patient with tuberous sclerosis.

The most important current reference book on TSC is undoubtedly Gomez' monograph on TSC, considered indispensable for clinicians and researchers in TSC (Tuberous sclerosis 1979 and 1988 (2nd ed); Tuberous Sclerosis Complex 1999). Examples of well-written reviews on the clinical aspects of tuberous sclerosis are those by Weiner et al.(1998), Harrison and Bolton (1997), and Griffiths and Martland (1997).

1.2.2 Prevalence of Tuberous Sclerosis Complex

Estimates on the prevalence of tuberous TSC have varied between 1:150,000 (Stevenson and Fischer 1956), and 1:9704 (Wiederholt, Gomez, and Kurland 1985). Population-based studies as well as studies in institutions of the mentally handicapped have been performed and only provided an overall impression or rough estimate of the true frequency of TSC in the general population. These kinds of studies resulted in an estimated prevalence of TSC at birth of around 1:10,000 and under five years of age of 1:15,000 (Hunt and Lindenbaum 1984). Under the assumption that the frequency of TSC is roughly equal across all ethnic groups, these figures would lead to an estimated current total number of 1,000-1,500 TSC patients in the Netherlands, and about 20-30 births with TSC per year. These figures should however be interpreted with caution, since in addition to the limitations of extrapolation there is insufficient knowledge about the survival of patients with TSC, whereas presumably the underdiagnosis of mild cases is also an important factor.

1.2.3 Clinical manifestations

Neurological signs

The neurological problems, predominantly consisting of epilepsy and mental retardation, represent the most severe and incapacitating expression of TSC. Epilepsy occurs in about 70% of patients at some point in life and is often difficult to control. When the onset of epilepsy is before the age of one year, a large proportion of these patients has impaired mental function. Mental retardation is present in just over 50% of TSC patients (unpublished data) and only occurs in the group with (past) epilepsy, of whom about two thirds has retardation. Controlling the epileptic seizures is considered quite important, in an attempt to reduce the development of "epileptic pseudodementia", the worsening of mental retardation as a consequence of repeated and severe epileptic seizures (Gomez 1999). Apart from the success of treatment of the infantile spasms with vigabatrin (Sabril®), improvement has been reported following a neurosurgical approach in selected cases with unequivocal identification of the source of epileptogenic activity (Baumgartner et al. 1997). Cranial imaging is recommended when a diagnosis of TSC is made or suspected. Currently, a CT-scan is considered to be the most reliable means to identify paraventricular subependymal nodules, often showing some degree of calcification. MRI-scan is to be preferred for studying the presence or absence of (sub)cortical tubers and is particularly useful in young children, in whom often the paraventricular nodules have not yet calcified. Over 90% of patients show subependymal nodules, cortical tubers or both (Nixon et al. 1989).

Skin abnormalities

In TSC, a wide variation of skin abnormalities can occur. Hardly distinguishable white spots, a mild shagreen (chagrin) patch or single subungual fibroma may be the only skin sign. On the other hand, angiofibromas, fibrous forehead plaques or ungual fibromas can be severe, seriously disfiguring and quite a problem for both patient and dermatologist or plastic surgeon. White spots or hypomelanotic macules are thought to occur in over 90% of TSC patients, and represent an early albeit not pathognomonic sign, especially suggesting a possible diagnosis of TSC when noted in a child with early onset epilepsy. In the Caucasian population, hypomelanotic spots can easily be missed on routine physical examination, and they are better visible when examination of the skin is done in a dark room with an ultraviolet Wood lamp (wavelength 360 nm). Variation in pigmentation, and the contrast between pigmented and hypopigmented areas of skin tends to increase with age. Therefore investigations with the Wood lamp is probably of less discriminative value in adult subjects.

Eyes

The most predominant and characteristic eye sign is the retinal hamartoma, often located in the periphery of the retina, and therefore asymptomatic. About 25%-50% of patients show this sign, which can sometimes be quite important for confirming the diagnosis of TSC (Zimmer-Galler and Robertson 1995). When accidentally discovered, retinal hamartomas have been

misdiagnosed as retinoblastoma, leading to enucleation of the eye in a patient not previously diagnosed with TSC. In principle, retinal hamartomas are benign, rarely grow and do not need treatment. Other ophthalmologic lesions reported in association with TSC are retinal pigment alterations, strabismus and coloboma of the iris. However, some of these symptoms may represent chance findings, rather than true associations.

Kidneys

The most distinguishing renal feature in TSC is the combination of multiple cysts and angiomyolipoma, a benign tumour of a mixed type. The presence of fat in this tumour, best demonstrated with a CT scan, distinguishes angiomyolipoma from renal cell carcinoma. Although mostly benign and not metastasising, malignant angiomyolipoma with invasive growth may occur in TSC patients (Al Saleem et al. 1998). Isolated angiomyolipoma (thus without systemic TSC) occurs as a sporadic condition, with a strong preponderance of female patients (F:M ratio 5:1). In TSC-related angiomyolipoma, the predominance of female patients is less striking, but still present, as reported by Van Baal (1987) in a large series of 170 patients (F:M ratio 3.6:1). Other important renal lesions in TSC are cysts that, on ultrasound and X-ray imaging, can be as large and multiple as in adult type autosomal dominant polycystic kidney disease (ADPKD) and difficult to be distinguished from ADPKD. Although angiomyolipomas have a tendency for growth, especially during childhood, cysts can both appear and disappear with time (Ewalt et al. 1998). Pathological studies have shown no clear macroscopical difference between cysts in TSC and those in ADPKD. Under the light microscope a difference in structure of the epithelium lining the walls of the cysts has been reported, with a predominance of hypertrophic and hyperplastic cells in TSC tissue, creating a "distinctive and perhaps unique pattern" (Bernstein 1993). Presently it is not known whether there are differences in structure between cysts of patients with a TSC1 mutation and of those with a TSC2 mutation, as insufficient patients with known mutations have been studied. Loss of heterozygosity (LOH) of either of the two genes has been demonstrated in a proportion of angiomyolipomas, rhabdomyomas and to a lesser degree in brain lesions (Henske et al. 1996). In cases with a known constitutional mutation, the LOH always concerned the same locus. Frequencies and severity of the renal lesions seem to be roughly equal for patients with a small constitutional TSC1 or TSC2 mutation.

In a small proportion of TSC patients, adult type autosomal dominant polycystic kidney disease (ADPKD) is present as a second genetic disease (Sampson et al. 1997). Patients with this syndrome present with congenitally enlarged kidneys or cystic disease in childhood and their diagnosis of polycystic kidney disease may precede the diagnosis of TSC. This group of patients has a relatively poor prognosis regarding their renal function. In several of these patients, a large deletion of the short arm of chromosome 16 can be demonstrated by Fluorescent in situ hybridisation (FISH) analysis, disrupting both the TSC2 gene and the adjacent PKD1 gene in a 'contiguous gene deletion syndrome'. One patient with the TSC2-PKD1 deletion syndrome has been described in whom renal pathology was indistinguishable

from the patients described by Bernstein (1993), leading to the hypothesis that the patients described by Bernstein might also have a deletion comprising both the TSC2 and PKD1 genes (Sampson et al. 1997).

Heart

In the general population, congenital cardiac tumours have been reported to occur with a frequency of 15 in 10,000 pregnancies (Abushaban, Denham, and Duff 1993). Of those patients, about 60% are considered to represent rhabdomyomas, which occur about twice as often in male babies as in female babies (Holley et al. 1995). The proportion of TSC patients reported in a population diagnosed with cardiac rhabdomyoma varied between 50% and 80% (Harding and Pagon 1990; Webb et al. 1994). An important aspect of clinical relevance of cardiac rhabdomyoma is, that it is the earliest detectable expression of TSC and may be visualised by foetal ultrasound scanning during pregnancy. Therefore, when in a pregnancy a child is diagnosed with an intracardiac tumour, one should be aware of the possible diagnosis of TSC. Follow-up investigations at birth and in the first year of life should be provided to investigate the possibility of TSC. Examination of the parents for mild expression of the disease and a family history may even reveal previously undiagnosed familial TSC. In our own experience, there have been a few children with congenital cardiac tumours, probably rhabdomyomas, that could only be proven to be TSC patients after a repeated dermatological evaluation in the first year of life. At an initial screen for signs of TSC shortly after birth, skin investigations using Wood lamp, ophthalmologic examination, cranial and renal ultrasound examination can all be normal.

In series of TSC patients unselected for cardiac symptoms the prevalence of cardiac rhabdomyoma varied between 30% (Tuberous sclerosis 1988), and 47% (Jozwiak et al. 1994). In case one of the parents is affected with TSC, the finding of a cardiac tumour in the foetus is very suggestive of the diagnosis of rhabdomyoma as part of TSC. Cardiac rhabdomyomas are associated with increased perinatal and postnatal mortality. The chance of cardiac insufficiency is probably related to the size and the site of the rhabdomyomas. Follow-up ultrasound imaging studies on the natural course show that these tumours usually become smaller, and sometimes even disappear completely. The mechanism of this spontaneous regression with age is presently unknown. Possibly restoration of the normal heart anatomy is aided by the fact that many cardiac cells are dinucleated and form functional syncitia, making them possibly less sensitive to gene dosage effects. Hormonal factors (female steroid levels and/or expression of steroid receptors) possibly play a role, as has also been proposed for pulmonary lymphangioleiomyomatosis, a TSC complication that occurs only in adult female TSC patients and is sometimes treated with anti-estrogens (unfortunately often with limited success).

Sometimes rhabdomyomas lead to disturbances of the heart rhythm, like Wolf-Parkinson-White syndrome (pre-excitation syndrome in patients with paroxysmal tachycardia), which

occurs about ten times more often in patients with TSC than in the general population and usually presents in the neonatal period (O'Callaghan et al. 1998).

Lungs

TSC of the lungs in the form of lymphangioleiomyomatosis (LAM) is rare and is almost uniquely present in women. LAM occurs in about 1%-2% of women with TSC and almost exclusively above 20 years (Tuberous sclerosis 1988). On chest X-ray a so-called 'honey comb' appearance of the lungs can be seen. On a CT scan, the lesions are characterised by the presence of small cyst like structures combined with fatty and smooth muscle tissue. LAM is traditionally associated with a poor prognosis, with death predicted within five years after diagnosis. However, in our experience, some patients with pulmonary TSC have a rather slower progression of their lung disease. Treatment can be given using anti-estrogenic therapy (e.g. tamoxifen), in order to delay progression of the lesions into emphysema and pulmonary insufficiency. The success of this treatment in some patients and the strong female preponderance of the lung problems in TSC patients, suggest that the development of LAM, like that of cardiac rhabdomyoma, are partly steroid-dependant or steroid-sensitive.

Oral cavity

On inspection of the oral cavity two types of lesions may indicate TSC (Tuberous sclerosis 1988). Firstly, gingival fibroma is the buccal equivalent of facial angiofibromas, to be differentiated from gingival hypertrophy induced by phenytoin, an epileptic drug that is frequently used by TSC patients with epilepsy. Secondly, on the teeth, especially on the flat surfaces of the incisors, small enamel pits or small circumscribed patches of hypoplasia can be seen, that can give rise to an increased sensitivity to develop caries. These enamel pits are also seen in unaffected individuals, with a reported prevalence of 7% (Mlynarczyk 1991), but a number of over fifteen pits is not seen in unaffected individuals (unpublished own data from a subset of unaffected relatives and partners of TSC patients). Enamel pitting can sometimes be a helpful sign to support the diagnosis in a patient with an uncertain diagnosis. Rarely, a relative of a patient with TSC is seen in whom prominent enamel pitting is the only detectable possible manifestation of TSC and no definite conclusion with respect to the diagnosis of TSC can be drawn.

Conclusion

In conclusion, it can be said that the prevalence of organ-specific symptoms is not only dependent on age, but also type, timing and frequency of the examinations. The best example of how the timing of examination may influence the prevalence estimate is probably cardiac rhabdomyoma (see table 1.2 and Chapter 2.1.2).

1.2.4 Diagnostic criteria of TSC, clinical signs and differential diagnosis

Diagnostic criteria for TSC

The clinical spectrum of TSC is very diverse and variable, with large differences in severity of the disease both between unrelated sporadic patients and between affected members in a single family. In view of the wide clinical variability of TSC, diagnostic criteria were proposed by Gomez (Tuberous sclerosis 1988), and revised several times (Gomez 1991; Roach et al. 1992; Roach, Gomez, and Northrup 1998; Tuberous Sclerosis Complex 1999).
In the last consensus report, diagnostic criteria were divided in two groups: major and minor features (Table 1.1.).
For a diagnosis of TSC, at least two major features should be present or one major and two minor features. With this revision of the diagnostic criteria, no single feature of TSC retained the attribute "pathognomonic". Since it is unclear whether this proposition was based on the virtual absence of TSC with monosymptomatic expression of pathognomonic signs, or on the existence of non-TSC diseases with such a symptom, the discard of pathognomonic symptoms is questionable.
In the majority of cases there can be no doubt about a diagnosis of TSC, even in a sporadic patient. Many debates arise about minimally affected relatives or a person with a monosymptomatic manifestation of TSC. As the occurrence of somatic mosaicism associated with intermediate phenotypes appears to be rather frequent (Chapter 3.7), comprehensive definition of the clinical spectrum might be impossible.

Prevalences of symptoms in TSC

Accurate data on the prevalence of the different signs and symptoms of TSC are scarce, as no extensive studies including the age of onset or clinical manifestation of the different symptoms of TSC have been published. All larger data sets are subject to various kinds of bias. Most studies are either hospital-based or population-based. Data of two representative studies are summarised in table 1.2, and compared with our own series (more extensively presented in Chapter 2). The Gomez study represented patients seen at the Mayo clinic, and therefore mainly included patients with symptoms leading to a referral to the clinic (Tuberous sclerosis 1988; Gomez 1991). Webb (1996) initiated a population survey in the South West of England and detected 122 patients (Webb, Fryer, and Osborne 1996). The patients in the study by Webb were identified through medical records and subsequent examination of close relatives, where possible.
In our own series, a heterogeneous group of patients is presented that was initially collected over a period of 10 years as part of the effort to identify the TSC genes. This group could be best characterised as a "clinical genetics" population of patients and includes all sporadic patients and index patients and in case of familial TSC also their affected relatives.

Table 1.1: Revised diagnostic criteria for the diagnosis of TSC (Roach et al., 1998)

Major features

1. Facial angiofibromas or forehead plaque
2. Non-traumatic ungual or periungual fibroma
3. Hypomelanotic macules (three or more)
4. Shagreen patch (connective tissue nevus)
5. Multiple retinal nodular hamartomas
6. Cortical tuber[a]
7. Subependymal nodule
8. Subependymal giant cell astrocytoma
9. Cardiac rhabdomyoma, single or multiple
10. Lymphangioleiomyomatosis[b]
11. Renal angiomyolipoma[b]

Minor features

1. Multiple, randomly distributed pits in dental enamel
2. Hamartomatous rectal polyps[c]
3. Bone cysts[d]
4. Cerebral white matter radial migration lines[de]
5. Gingival fibromas
6. Non-renal hamartoma
7. Retinal achromic patch[c]
8. 'Confetti' skin lesions
9. Multiple renal cysts[c]

Definite Tuberous sclerosis complex: Two major features or one major plus two minor features
Probable tuberous sclerosis complex: One major plus one minor feature
Possible tuberous sclerosis complex: Either one major or two minor features

[a]When cerebral cortical dysplasia and cerebral white matter migration tracts occur together, they should be counted as one rather than two features of TSC

[b]When both lymphangioleiomyomatosis and renal angiomyolipomas are present, other symptoms of TSC should be present before a definite diagnosis is assigned

[c]Histological confirmation is suggested

[d]Radiographic confirmation is sufficient

[e]One panel member (MRG) felt strongly that three or more radial migration lines should constitute a major sign

Comparison of the three studies shows that the hospital/outpatient clinic based study by Gomez shows high scores for epilepsy, probably because epilepsy is one of the referral symptoms. The proportion of retinal hamartomas is high, possibly because these can be difficult to detect and the centre at the Mayo clinic has good expertise. The figures of Webb and our own study that is based on data from multiple different sources are then probably too low. Rhabdomyomas are found in high proportion in the Mayo series, as it represents a comparatively young paediatric population, with many children with infantile spasms, mostly occurring under the age of one year. This fact also would explain comparatively low figures for angiofibroma and ungual fibromas. Percentages for mental retardation, giant cell astrocytoma and presence or absence of Vogts triad are comparable for the Gomez study and our own data. The Webb study shows similar figures for all parameters, which suggests that our data are reasonably representative of a population-based set.

Table 1.2: Large series of the prevalence of symptoms of TSC

Symptom	Gomez 1988/91 (n=300)	Webb 1996 (n=122)	Our series (n=370)
Type of study population	*Pathologic/paediatric neurologic*	*Population survey*	*Clinical genetic*
Epilepsy (overall)	92%	78%	74%
Age at first seizure <1y		70%	44%
Age at first seizure 1y-5y		16%	21%
Age at first seizure 5y-15y		9%	20%
Age at first seizure >15y		4%	15%
Mental retardation (MR)	48%	53%	54%
Subependymal giant cell astrocytoma	6%		5%
Angiofibroma	56%		71%
Ungual fibromas	18%		30%
Hypomelanotic macules	90%		77%
Retinal hamartomas	47%	25%	19%
Rhabdomyomas (overall)			9%
Age at documentation 0-1y			57%
Age at documentation 0-6y	64%		13%
Vogts triad (epilepsy + MR + angiofibroma)	29%		36%
Vogts triad, all signs absent	6%		9%

Differential diagnosis

A number of conditions may be confused with TSC depending on the presenting signs in the patient. Some examples are shown in table 1.3.

On CT scan of the brain, skull X-ray or pneumencephalogram, paraventricular nodules can be misinterpreted as signs of cerebral arteriovenous malformations (Tuberous sclerosis 1988), or

a congenital infection, especially toxoplasmosis and cytomegaly. MRI scan pictures may cause confusion with familial periventricular nodular heterotopias (OMIM 300049), an X-linked condition occurring in girls and women only, and supposed to be lethal in most male conceptuses (Chapter 2.1).

Facial angiofibroma can mimic acne vulgaris or acne rosacea or miliae, but is also easily confused with a mild manifestation of the hereditary multiple epithelioma/ cylindromatosis spectrum (OMIM 132700, Chapter 2.5). Multiple ungual fibromas used to be considered pathognomonic for TSC and not to be confused with any other condition, except perhaps the rare generalised familial 20-nail dysplasia (OMIM 161050). Single ungual fibroma can also arise spontaneously or result from fibrosis by the scarring of a damaged nail bed.

The retinal lesions of TSC are sometimes mistaken for retinoblastoma (and vice versa) or can mimic Drusen, as can be found also in diabetes mellitus. Congenital cardiac tumours are rare, and in most cases are either rhabdomyomas or myxomas. Lymphangioleiomyomatosis (LAM) of the lungs also may appear as an isolated condition but in some patients LAM is combined with renal angiomyolipoma, without other evidence for TSC. It is presently unknown whether these cases should be viewed as mono- or oligo-symptomatic manifestations of TSC, possibly as a consequence of a somatic mutation. In support of this theory, in angiomyolipomas of patients with LAM loss of heterozygosity of the TSC2 region was demonstrated (Smolarek et al. 1998), but no germ line mutations could be found in the TSC2 gene of these patients (Astrinidis et al. 2000). As only half of the lesions showed LOH of TSC2, other genes could be involved as well.

Renal angiomyolipoma must be differentiated from renal cell carcinoma that does not contain fatty tissue. Polycystic kidneys can also be a sign of the adult type polycystic kidney disease.

Enamel pitting or hypoplasia is one of the minor criteria of TSC, but can also be found in normal healthy subjects. The localisation and shape of gingival fibromas of TSC can easily be distinguished from the much more generalised gingival hyperplasia that can occur as a separate clinical condition, or as a result of chronic phenytoin medication.

Most of the TSC manifestations can be encountered as an isolated symptom, but in general, autosomal dominant inheritance of monosymptomatic TSC lesions is not seen. It is therefore unlikely that such lesions represent 'mild germ line mutations' of the TSC1 or TSC2 gene, assuming that such a mild mutation would not affect reproductive fitness. An explanation for single lesions might be the occurrence of segmental somatic mosaicism in an oligo-symptomatic individual, followed by a chance 'second hit' event.

Table 1.3: Presenting signs of TSC at different ages and depending on the site of the first major manifestation of TSC, with their differential diagnosis

Age	Sign	Differential diagnosis
Pregnancy/newborn	Intracardiac tumour	Rhabdomyoma
		Myxoma
	Intracerebral calcifications	Cerebral arteriovenous malformation
		Congenital Infection: e.g.
		Toxoplasmosis
		Cytomegaly
	Cortical tubers	Isolated, focal, cortical dysplasias
Infants/toddlers	Epilepsy/infantile spasms	Symptomatic epilepsy
		Idiopathic epilepsy
	Hypomelanosis	Hypomelanosis of Ito
	Epilepsy, mild mental retardation, MRI abnormalities	Familial periventricular nodular heterotopias (X-linked)
	Facial angiofibroma	Cylindromatosis/epitheliomas
		Acné rosacea
		Milia
	Retinal tumour	Retinoblastoma
Children	Epilepsy, moderate to severe mental retardation	Dysmorphology syndrome
		Congenital infection
		Post-anoxic encephalopathy
(Young) adults	Renal problems	Polycystic kidney disease
		Malignancy (renal cell carcinoma)
	Retinal tumour	Drusen (e.g. from diabetes)
	Honeycomb lung (X-Ray)	Isolated lymfangioleiomyomatosis

1.2.5 Genetics and genetic counselling of Tuberous Sclerosis Complex

The TSC1 and TSC2 gene:

A mutation in either the TSC1 gene on chromosome 9 or the TSC2 gene on chromosome 16 causes TSC. The TSC1 gene spans 45 kb of genomic sequence and exists of 23 exons. The 8.6 kb mRNA transcript encodes an 1164 amino-acid long protein, with an estimated molecular mass of 130 kDa, named *hamartin* (van Slegtenhorst et al. 1997). The TSC2 gene covers 45 kb of genomic sequence and has 41 exons, encoding a transcript of about 5.5 kb. The protein is named *tuberin* and has a length of 1807 amino-acids and a molecular mass of about 200 kDa (The European Chromosome 16 Tuberous Sclerosis Consortium 1993). *Hamartin* and *tuberin* show no homology. The predicted coiled-coil domain of *hamartin* was shown to interact with *tuberin*. In *tuberin* a so-called GAP domain was recognised, that might be involved in a tumour suppressor function of the gene (van Slegtenhorst et al. 1997). For both the TSC1 gene and the TSC2 gene, loss of heterozygosity in tumour tissue has been shown, supporting the hypothesis that they both have a tumour suppressor function (Henske et al. 1997; van Slegtenhorst et al. 1998).

DNA-diagnosis of TSC

About one third of patients represent familial cases with more than one individual in a family diagnosed with TSC. Up to two thirds of the cases are thus considered due to a 'new mutation', and occur outside a family context of TSC. Due to the complexity of the genes, and the fact that almost each family has a unique mutation, the identification of mutations is laborious. Several techniques have to be used in mutation analysis of the genes involved in TSC, in order to achieve a 90% detection rate. Fluorescent In Situ Hybridisation (FISH) analysis and Southern blot analysis are used for the detection of whole gene deletions and other large rearrangements in the TSC2 genomic region. For the detection of smaller mutations in both the TSC1 and TSC2 gene, Single Strand Conformation Polymorphism (SSCP) or Denaturing Gradient Gel Electrophoresis (DGGE) analysis is used. PCR products of fragments giving an abnormal pattern in the analysis, are sequenced. The Protein Truncation Test (PTT) for the detection of truncating mutations is not operational for mutation analysis of the TSC genes in our laboratory.

When an abnormality is detected, usually a blood sample from both parents is requested for comparison of the DNA-results in order to make the distinction between a pathogenic mutation and a polymorphism and to evaluate the possible presence of parental somatic mosaicism. Allele specific oligonucleotide hybridisation (ASO) analysis is a good method for this comparison, as it gives clear cut test results and is easy to use. In order to rule out common polymorphisms (Cotton and Scriver 1998), missense changes are compared with 400 control alleles from 200 random DNA samples (non-TSC patients). In addition, mutation analysis and clinical examination in the parents may help to discriminate between a polymorphism and a disease causing mutation. Subsequently, requests for carrier detection by

other family members can then be offered. With the employed methods, screening of all exons of the TSC1 gene and about a third of the exons of the TSC2 gene has resulted in the detection of a mutation in 30% to 40% of the index patients
An overview of the mutations we detected in the TSC1 and the TSC2 gene is presented in Chapter 3. In the TSC1 gene, the vast majority of mutations are truncating mutations, whereas larger deletions or insertions have rarely been reported. In the TSC2 gene, different types of mutations have been described: large deletions, insertions, non-sense, missense, frame-shift and splice-site mutations. Mutations are detected throughout each gene, without clear hot spots of mutation, although recurrent mutations have been described (Au et al. 1998; Verhoef et al. 1998; Beauchamp et al. 1998).

Genetic counselling for TSC

Tuberous sclerosis complex is an autosomal dominant disease, with a high penetrance and a variable pattern of clinical and subclinical expression. TSC is a complicated condition, both clinically and genetically, so ideally the counselling of the families should be provided by clinicians with ample experience with the condition. For families with a child with TSC, a lot of time and effort is needed to give the proper information and support. It is important for them to come to grips with a very complicated disease and to understand the natural variation between patients within one family. Much of the input can be provided by specialised clinicians: paediatricians, paediatric neurologist and clinical geneticist, whose role also is to pass information to the primary care worker, e.g. the general practitioner. At the time of discussing the reproductive decisions, formal genetic counselling is recommended, for reasons of the complexity of this genetic condition.
For a long time, the clinical variability of the disease was considered to be independent of the mutated gene or the type of causative mutation involved. The clinical expression of TSC depends therefore also on factors outside the mutated gene.
Despite our progress in knowledge of the mutational spectrum, it is still hard to understand the difference in proportion of patients with a detected TSC1 mutation in familial cases (about 50%) and in sporadic patients (about 20%). A possibly contributing factor could be reduced reproductive fitness in TSC2-related disease compared to TSC1-related disease because of more severe disease in the former. Initially in studies focussing on TSC families, no significant difference could be demonstrated in clinical severity between TSC1- and TSC2-related disease. However, larger studies with a higher proportion of sporadic patients did indicate that TSC1-related disease is comparatively 'milder'(Dabora et al. 2001; Jones et al. 1997). Such studies are hampered by the less frequent occurrence of sporadic TSC1-related disease. The patients with a 'contiguous gene deletion phenotype' of TSC, about 5 % of patients form a more or less separate entity, and have severe (mostly congenital) polycystic kidney disease and TSC. These patients have a large deletion encompassing both the TSC2 gene and adjacent PKD1 gene, disrupting the function of both genes (Sampson et al. 1997).

When a patient is diagnosed with certainty as affected with TSC, and parents or other relatives request genetic counselling, mutation analysis is recommended. When a mutation is identified in an index patient, parents can be screened for the presence or absence of the mutation. If a TSC patient is a somatic mosaic, implying a post-zygotic origin of the mutation (apart from reversal of a mutation, which is exceptionally rare), mutation analysis of parents and sibs is not necessary. When the mutation is not known, or it is unknown whether a presumed 'de novo' mutation has arisen pre- or post-zygotically, parents of a child with TSC should have a full physical examination, with a focus on skin abnormalities. A normal DNA result in both parents is insufficient proof of a 'de novo' mutation in the child, since a mutation for which one of the parents is mosaic may be confined to tissues that were not examined, or the degree of mosaicism might be below the detection limit. Thus clinical investigations remain necessary to detect possible signs of somatic mosaicism in the parents. In some families multiple occurrences among offspring of clinically unaffected parents have been demonstrated to be due to germ-line mosaicism in one of the parents, see also Chapter 3.7 and (Yates et al. 1997; Rose et al. 1999). A full clinical examination as advised in consensus consists of the following items (Roach et al. 1999):

1. Investigations of the skin, with an emphasis on the possible presence of white patches, ungual fibromas and facial angiofibroma.
2. Neurological examination, with neuro-imaging by CT- scan or MRI- scan of the brain, in order to detect subependymal calcified nodules, cortical tubers and radial migration lines. MRI- scanning is more suited to detect white matter migratory lesions and cortical tubers, whilst the CT- scan is a more reliable tool to detect calcified paraventricular (subependymal) nodules. As these are probably more specific at the adult age, CT-scanning is the preferred method of investigation in adults, MRI- scanning would be more suitable at younger age, as calcification of TSC lesions generally occurs after the first years of life.
3. Ophthalmologic screening, with emphasis on retinal hamartomas which can be located especially in the periphery of the retina. Dilatation of the pupils prior to the retinal examination is therefore recommended.
4. Abdominal ultrasound to screen for renal cysts and/or angiomyolipomas, or cysts or angiomyolipomas of the liver or pancreas.
5. Cardiac ultrasound for intramural rhabdomyomas.
6. Oral examination with a focus on enamel pits and/or gingival fibromas.

Debate has arisen whether the full clinical screen is mandatory under all circumstances. For example when screening parents for signs and symptoms of TSC, cardiac examination probably has no contributory value for the diagnosis. It is estimated that about ±98% of carriers of TSC will be diagnosed by investigation of the skin, CT- scan of the brain and ophthalmologic evaluation. Renal ultrasound scanning has clinical implications if an

abnormality is found, and is therefore also recommended. Once a possible sign of TSC is found, full examination should be completed. It is advised to examine parents of a patient first of all, but the same examination scheme is advisable to sibs of the index patient, who want to exclude the risk of TSC as much as possible, even after normal results in the parents. In case of clinically unaffected parents of a child with TSC the estimated chance of recurrence in a further child is about one percent. In families in which the causative mutation is known, DNA-testing can replace most but not all of the clinical investigations. In sibs and children of a patient DNA testing is sufficient, as they can not have mosaicism.

For pregnancies in families with a known mutation, prenatal DNA-analysis can be offered on chorionic villus cells as well as amniotic fluid cells, and the possibility of an affected child as a result of undetected gonadal mosaicism of one of the parents can be investigated. If the mutation is not known, and if there is no significant linkage with either one of the two TSC loci, the structural ultrasound screening can be offered, from about the 16th week of pregnancy. However, it seems advisable to reserve this method for high risk pregnancies only, and parents should be informed about the limited predictive value of the method in detecting TSC in the second trimester of pregnancy. Often rhabdomyomas will become visible only after the 24th week. Even though, detection of the foetal cardiac condition at a later stage of pregnancy could be of benefit in preparation of delivery and postnatal care. Because of the limited value of prenatal ultrasound before the 24th week, it is questionable whether foetal monitoring should be offered in low prior risk situations.

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CHAPTER 2

PHENOTYPES OF TUBEROUS SCLEROSIS COMPLEX

2.1
Introduction

As alluded to in the previous chapter, insight in the full spectrum of TSC-related problems is limited. Most of the available information is derived from retrospective databases with data of patients reported by clinicians and researchers. This implies that the ascertainment of patients with TSC as well as the description of the TSC patients within the database is far from complete. The current patient database as described in Chapter 2.2 comprises the clinical data only of patients with a certain diagnosis of TSC, according to the revised criteria of Gomez (Roach et al. 1998). Despite attempts to gather complete information on these patients, the sources from which the data have been collected were necessarily incomplete. A long-term systematic and therefore labour-intensive follow/up of identified patient groups would be mandatory in order to obtain a more accurate picture of ages at onset and true frequencies of symptoms and complications. Many of the clinical manifestations of TSC are stationary or slowly progressive, and are more recognised for their contribution of the clinical diagnosis of TSC than for their medical impact in terms of the course of the disease or complications. However, some of the lesions caused by TSC can cause severe problems, amongst which are: 1) the congenital cardiac abnormalities, 2) the epilepsy including the related mental retardation and behavioural problems, 3) the development of subependymal giant cell astrocytoma and 4) the complications of renal angiomyolipomas and cysts.
During our study period, several remarkable case and family histories were presented to us, involving clinical problems concerning the management of (apparently) TSC-related issues, examples of which are presented at the end of this section and in the published Chapters 2.3, 2.4 and 2.5. Clearly, concentration of expertise concerning rare diseases like TSC may have direct benefits to the patients with the (suspected) disorder, especially in situations of unusual complications or medical histories.

Malignant disease in TSC

Invasive malignant disease is rare in TSC patients, as the tumours related to TSC are from hamartomatous origin and do not grow invasively nor metastasize. Some associations of TSC with malignancies have been reported anecdotally, but were not supported by epidemiological studies. Two malignant tumour types encountered in TSC patients, as reported in Gomez (1999), are renal cell carcinoma and oncocytoma. Thyroid adenoma possibly occurs more often than expected in TSC patients. Recently, somatic mutations in the TSC1 gene have been described in bladder tumours (Hornigold et al. 1999). For most lesions affecting the internal organs, the medical complications result from a tendency to vascular rupture in the lesion (tumour) or haemorrhage following resection. The blood vessels, which are part of the renal angiomyolipomas for example, may be subject to severe bleeding, and this is an important cause of premature mortality in TSC patients with renal manifestations of the disease. Mortality related to subependymal giant cell tumours in the brain of TSC patients is more

often caused by postoperative haemorrhage after (partial) resection of the tumour than by local damage from invasive growth.
In Chapter 2.3, a boy is described with TSC who developed an unusual malignancy. He was a mentally retarded boy with severe TSC, who presented with an abdominal mass of unknown origin. Exploratory surgery was needed in order to demonstrate that the tumour was a malignant infiltrating pancreatic tumour of mixed endocrine origin, although not actively hormone producing. Loss of heterozygosity (LOH) studies of the tumour tissue showed loss of the TSC2 gene region on chromosome 16 and subsequently the TSC2 germ-line mutation (the first hit) in the TSC2 gene was shown. Absence of the wild type TSC2 allele in the tumour was interpreted as the second hit and suggests a relationship between the tumour and TSC. Mixed type neuro-endocrine tumours have previously been associated with multiple endocrine neoplasia syndrome type-1 (MEN-1 syndrome). However, other signs of MEN-1 syndrome were absent in the patient and, in contrast to the normal situation in MEN-1 syndrome, in our patient the tumour was not hormonally active. Islet cell tumours have been reported before in TSC patients, but were discovered after unexplained hypoglycaemia periods rather than through mass effects.
In conclusion, malignancies are rather rare in TSC, but when seen in a TSC patient involvement of one of the TSC genes in their pathogenesis has to be considered.

Follow-up studies

The material we collected during our study is not suited for comprehensive follow-up studies of symptoms of individual patients, as the data tend to be cross-sectional rather than longitudinal over a number of years. Reliable data on a large group of TSC patients can only be gathered from specialised clinics for TSC patients of all ages addressing the whole spectrum of symptoms and signs comprehensively by regular follow-up, care and consultation. Such initiatives will eventually help to develop, adjust and improve the protocols for follow-up examinations aiming at prevention. In some countries, like the UK and the Netherlands, such clinics have been initiated recently and will hopefully generate follow-up data in due time.
It is recognised that in adult TSC patients, the severe complication of retroperitoneal haemorrhage in renal lesions represents the major cause of mortality, and that therefore larger renal lesions need preventive treatment. Large studies providing detailed follow-up information on the general course of renal TSC are lacking. In Chapter 2.4 we present a small clinical follow-up study of renal complications of TSC, and this study represents one of a few reports in which TSC patients have had a semi-prospective 5-year follow-up monitoring the progression in their renal lesions. The results of this study confirm that renal problems account for substantial morbidity and premature mortality in TSC patients. In the article, a recommendation was formulated that large angiomyolipomas (of a size larger than 35mm) should be preventively treated by e.g. embolisation, as they are particularly prone to sudden severe haemorrhage.

Cardiac rhabdomyomas are most frequently detected in pregnancy, neonatally or in the first months of life. If heart rhythm disturbances are mild or absent, these children usually have a good prognosis (Smith et al. 1989; Smythe et al. 1990). Often the haemodynamic importance of the intracardiac tumours diminishes with time and with the growth of the heart muscle, and the effects on heart rhythm subside. However, in our study population, 4 of the 23 patients who died prematurely were children who died in the first month of life from problems related to congenital cardiac rhabdomyoma. In the literature, successful heart transplantation has been described for at least one TSC patient (Demkow et al. 1995).

The management of epilepsy in TSC patients is complex, as often the spectrum of epileptic seizures is wide, varies considerably with age, whereas the epileptic seizures in TSC tend to be refractory to anti-epileptic drugs (Fukushima et al. 1998). Infantile spasms are among the presenting signs of TSC in about two thirds of patients, with a median age of onset of 4 months (range 1 to 18 months). After the age of 1 year partial epilepsy with complex partial seizures, with or without secondary generalisation, is the most reported seizure type. At adult age, epilepsy is also frequent but rarely the presenting sign of TSC (Tuberous Sclerosis Complex 1999). If adult patients present with sudden neurological problems, the possibility of a giant cell astrocytoma must be considered. In some publications, a pro-active follow-up of patients with subependymal nodules close to the foramen of Monro is advised. Early surgical intervention, preferentially under stereotactic guidance can prevent further structural and functional damage, improve seizure control and prevent the development of hydrocephaly (Torres et al. 1998; Di Rocco, Iannelli, and Marchese 1995).

Differential diagnosis of TSC

As described in section 1.2.4, the clinical spectrum of TSC is diverse. The list of possible alternatives for the diagnosis TSC varies with the presenting signs and the age at presentation, especially when the signs are rather aspecific. In Chapter 2.5 and at the end of this section two histories are presented of 'TSC families', who in fact had a different hereditary disease.

Cylindromatosis

Chapter 2.5 reports a family referred with the initial diagnosis of TSC with isolated 'severe facial angiofibroma'. The family condition turned out to be an autosomal dominant cylindromatosis. In retrospect, the correct histological diagnoses of epithelioma and cylindroma had been made in some of the removed pathological tissue, but the individual descriptions had not been translated into a family perspective of a hereditary disease. Instead, the pathological changes had been interpreted to fit into the TSC spectrum. Linkage analysis excluded the involvement of both TSC chromosomal regions (chromosomes 9q and 16p) and revised clinical diagnosis was in agreement with cylindromatosis. Subsequent investigations demonstrated loss of heterozygosity (LOH) of chromosome 16q and a LOD score of over 3 with the candidate region of the cylindromatosis gene CYLD1 which confirmed the diagnosis autosomal dominant familial cylindromatosis (MIM 132700) in this family. Although this

condition may have quite severe skin abnormalities, the family was partially relieved by information that epilepsy and mental retardation were not part of their hereditary condition. Recently, the CYLD1 gene was isolated (Bignell et al. 2000) and the family we presented was shown to have a mutation (Q857X) in exon 19 of the CYLD1 gene, causing a truncation of the encoded protein by the introduction of a premature stop codon.

Familial periventricular nodular heterotopia

A 7-year-old girl was referred to the department of clinical genetics for a second opinion on her epilepsy syndrome. She was the second daughter from healthy parents. The pregnancy and delivery had been normal. At birth she weighed 3000 grams and had Apgar scores of 9 and 10 after 1 and 5 minutes respectively. At the age of three weeks a slight asymmetry was noted in her leg movements and physical examination of her back revealed a small sacral dimple, under which an open vertebral arch was later shown by X-ray investigation. Following her first DTP vaccination at the age of about 3 months, she had a seizure with asymmetrical shaking and a fixed gaze. Her EEG at age 4 months was described as 'restless'. A subsequent CT scan of the brain showed no abnormalities. At age one, her heart was evaluated because of a murmur, and she appeared to have a patent ductus arteriosus. The ductus was blocked operatively by catheterisation. Her developmental milestones were achieved in time and she was of normal intelligence. MRI scanning of the brain at age 18 months revealed multiple periventricular heterotopias, resembling candle guttering as is seen in tuberous sclerosis complex. Other abnormalities noted were a thin corpus callosum and megacisterna magna. No cortical or subcortical tubers were visible. Following the MRI findings, subsequent investigations for signs of TSC were performed and showed no other abnormalities. Specific attention was paid to the possibility of hypomelanotic macules, facial angiofibromas, ungual fibromas, chagrin patches and fibrous plaques. Also renal ultrasound and ophthalmologic examination were done with normal results, apart from the signs already described above. Around her second birthday she had a more generalised seizure with postictal hemiparesis. Over time she developed partial seizures with secondary generalisation.

Her family history was negative for epilepsy or mental retardation. Both parents were seemingly healthy and had undergone clinical screening for signs of TSC, including CT scan of the brain. No abnormalities had been detected. MRI of the brain of the parents was not yet performed. Between the time of referral and her visit to our clinic, in a second neuro-radiologic opinion, the diagnosis of TSC of the brain had been questioned, and the lesions had been described as subependymally localised heterotopias of grey matter, but no diagnosis had been mentioned. As far as the family data are available, there is no increase in the number of miscarriages or stillbirths, but there is a preponderance of female gender among maternal relatives.

The clinical history and MRI images of this girl clearly fit the pattern seen in the condition familial periventricular nodular heterotopia (FNH). FNH is an X-linked dominant disorder with supposed lethality in the vast majority of affected male foetuses (Palmini et al. 1993;

Barkovich, Gressens, and Evard 1992; Fink et al. 1997). In families with this disorder an increased proportion of (male) pregnancies results in abortion or foetal death. Most of the affected (almost exclusively female) patients are of normal intelligence. Carrier females can be completely asymptomatic and are then only diagnosed by specific MRI imaging or through multiple affected offspring. The association of FNH with thin corpus callosum and megacisterna magna is known from the literature (Oda et al. 1993), and patent ductus arteriosus as well (Huttenlocher, Taravath, and Miojtahedi 1994). The neurological similarities and mode of presentation between FNH and some patients with TSC have been discussed in several papers (Kamuro and Tenokuchi 1993; Jardine, Clarke, and Super 1996; Raymond et al. 1994). Other neuronal migration disorders discussed in the differential diagnosis are diffuse cortical dysplasia, or double cortex syndrome (Palmini et al. 1991), familial cortical band heterotopias (DiMario et al. 1993).

FNH is linked to band Xq28 on the X-chromosome, making linkage analysis in large families possible. This disorder, however, will often be perceived as a milder condition than tuberous sclerosis complex, as the affected females are generally of normal intelligence. In practice therefore, prenatal diagnostic testing will probably not often be requested. The gene mutated in FNH, the FLN1 gene, was identified in 1998. Its product filamin 1 is involved in neuronal migration and fulfils an unknown but apparently essential role in embryogenesis of the brain (Fox et al. 2001).

Evidently the implications of the change of diagnosis in this family were considerable. Both the parents had been completely investigated for signs of TSC, including CT scan of the brain. All these investigations had shown normal results. As a consequence, the conclusion had been drawn that it was very likely that the daughter was a sporadic TSC patient, with a low recurrence risk estimate for the parents. Firstly, the finding of FNH implies that MRI screening should be done in the mother, as usually CT-scanning does not detect this condition. She might be affected with FNH as well and thus have passed the condition on to her daughter. Secondly, the one-year-older other daughter in the family has a 50% a-priori chance of being a carrier, presuming that her mother is a carrier. The son has a low chance to be a gene carrier, although at some stage MRI scanning or DNA-analysis might be considered useful for complete reassurance. In addition, the sisters of the mother are at risk to be carriers, and should be offered genetic counselling and MRI scanning if they want to know their carrier status.

2.2
The tuberous sclerosis complex registry

S. Verhoef, R. Verhage

In the period 1988-1998, a large clinical and molecular study was undertaken at the Department of Clinical Genetics at the Erasmus University Rotterdam and the University Hospital Rotterdam. The principal aim of the study was the identification of the gene(s) for Tuberous Sclerosis Complex (TSC) by collecting and analysing large families for linkage studies and as many patients as possible for mutation searches in candidate genes. Once the genes had been localised and characterised, mutation analysis became feasible and the collection of clinical data on families and sporadic patients with TSC was continued for the purpose of studying the important issue of genotype-phenotype correlations.

2.2.1 Materials and Methods:

Introduction

All clinical information from the TSC patients with a definite diagnosis of TSC according to Gomez' diagnostic criteria (1991) was entered into a database. For each patient with TSC, as much clinical data as possible were registered, clinical as well as subclinical manifestations of the disease. The reported age at the time of the diagnosis of TSC and the best approximation of the respective ages at documentation of the signs and symptoms of the disease were scored. Approval for this study had been obtained from the Medical Ethics Committee (MEC) of the Erasmus University Rotterdam and University Hospital Rotterdam.

Ascertainment of patients

Patients with TSC were ascertained through multiple sources, and phenotypic data on the same patient from different sources were compared for validation and combined into the database. The following sources contributed to the registry:

1. Families seen for genetic counselling at the Department of Clinical Genetics of the University Hospital Rotterdam.
2. Families originally collected in a previous tuberous sclerosis complex research project by the late Dr. P. Fleury, paediatric neurologist at the Amsterdam Medical Centre.
3. Families that were informed about the TSC research project through the Dutch patient society STSN (Stichting Tubereuze Sclerose Nederland). The project was announced and presented in the Society's Bulletin and at several of their meetings information was given on different aspects of TSC and about the progress of the research project.
4. Through 'cascades': i.e. family members, deciding to participate themselves, following contact with a relative already involved in the research project.

5. Patients referred to our department for expert advice or second opinion from other (academic) centres.
6. Patients seen elsewhere by e.g. paediatricians, clinical geneticists or neurologists.
7. Patients seen by the clinical geneticist during consultation visits in institutes for the mentally handicapped.
8. Patients referred through specialised epilepsy (outpatient) clinics.
9. Patients identified by members of the Sophia Children's Hospital working group on neurocutaneous disorders.

The study period covered successive and overlapping stages of the scientific research, beginning with the phase of initial linkage analysis and the cloning and characterisation of the TSC1 and the TSC2 genes. Then the attention became focussed on mutation analysis of the two genes and the study of the functions of the gene products, hamartin and tuberin respectively, along with the investigation of possible relations between genotype and phenotype. This sequence of study aims implies a potential change of the characteristics of the patients and families that were ascertained over the entire duration of our studies. Initially, large families suitable for linkage studies had been recruited. In the period of testing candidate genes, the focus was more on sporadic patients, because of a greater chance of identification mutations of candidate TSC genes with larger numbers of patients with a 'de novo' mutation. Finally, the identification of both genes for TSC made mutation analysis for diagnostic purposes possible, and clinicians from elsewhere sent in blood and data from patients from families requesting genetic counselling with the purpose of getting DNA-confirmation of the diagnosis.

Age at documentation of diagnosis, symptoms or signs

In order to study possible genotype-phenotype correlation and age-specific expression of signs and symptoms of TSC, the reported ages of onset or discovery of each of the symptoms and the actual diagnosis of TSC were recorded in the database. Given the retrospective nature of the acquisition of clinical data in this study, the age at onset of each symptom was usually difficult to establish. The age of onset of a symptom may be much earlier than the first recognition of it by the patient or the parents, the subsequent observation by the physician, and finally the diagnosis of TSC. Cortical tubers are a good example of the problems encountered in determining the age of onset: this abnormality develops during embryogenesis, whereas its actual presence might only be detected by neuro-imaging, or as an unexpected finding at post mortem examination. Thus, in this registry we grossly deal with four different kinds of 'age of onset':

1) Age of actual onset of a symptom.
2) Age of which the patient recognised the presence of a symptom.
3) Age of confirmation of a symptom or recognition of a sign by the physician.
4) Age of which the diagnosis of TSC was made.

For some symptoms, not only the presence, but also the absence at a certain age was documented. Mostly these data were obtained during a diagnostic evaluation or follow-up study of TSC patients, or at the time of diagnostic work-up of relatives referred for genetic counselling.

Since for many patients and symptoms not all the information was available, we computed the best approximation of the actual age at presentation by selecting one of these values according to the following hierarchical order:

- The date of an investigation that reveals the sign or symptom
- The date of discharge from the hospital, if a sign or symptom became apparent or was first noticed during a stay in hospital
- The date of the letter first reporting the symptom
- The date of presentation of a symptom according to a relative
- The age at death, if the sign/symptom was found at autopsy

In the case of uncertainty, a best estimate was made, e.g. if a symptom was reported as having occurred or presented during the first year of life, the age at presentation was arbitrarily set at one year.

Classification of epileptic seizures

For the classification of the epileptic seizure types, we applied the operational diagnostic criteria for epileptic seizures, as formulated during the Workshop held by the 'Concerted Action on Genetic Analysis of Epilepsy' group between 4 and 7 November 1993. These operational criteria are based on the ILAE (International League Against Epilepsy) classification of epileptic seizures. The clinical data used were mainly extracted from the medical reports by the (paediatric) neurologist or paediatrician. The descriptions of the epileptic seizures were reviewed and classified by two independent investigators.

2.2.2 Phenotypic variation in the TSC population

Introduction

By 1999, the Rotterdam clinical tuberous sclerosis complex registry contained data on 382 affected individuals. The 382 patients come from 274 families, 220 sporadic cases and 162 familial cases. The average number of affected patients per family was 3.0 (162 in 54 families).

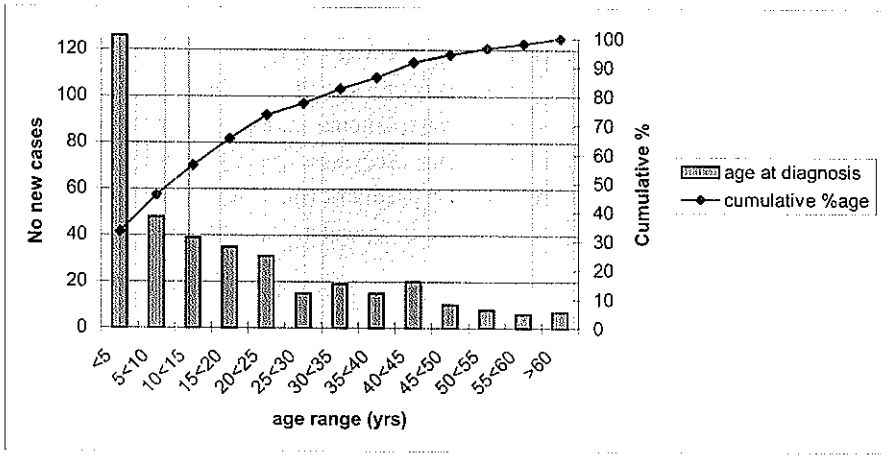
An overview is presented with data on all reported signs and symptoms in those patients. The male to female ratio was 180/202 (0.89). For a small number of symptoms the reported prevalence could be assumed to be close to the 'true prevalence' for the whole TSC

population, e.g. the symptoms of Vogt's triad: facial angiofibroma, epilepsy and mental retardation, as these symptoms are easily recognisable and documented. For 12 patients no data other than a "certain diagnosis of TSC" was registered, so they were not used for the phenotypic study. 10 of those 12 patients, the pathogenic mutation in one of the TSC genes was identified, confirming TSC as the correct diagnosis in the absence of clinical information. A total of 3861 items were scored present or absent for the remaining 370 patients: 195 female patients and 175 male patients (of which on average 7.4 symptoms per patient was scored as present, n= 2728).

Ages at diagnosis and documentation of the symptoms of TSC

Of all 382 patients in our data set, an age at diagnosis is given. The diagnosis of tuberous sclerosis complex was made before the age of five years in about a third of the cases. The second diagnostic tertile runs roughly from five to twenty years, and about one third of cases was diagnosed in the age group over twenty (Figure 2.1). Some cases of late diagnosis were evidently the result of cascade screening in families, in which the index case is a younger person, often with a more severe type of TSC and subsequently one of the parents was found to be affected, with a milder or less obvious expression pattern.

Figure 2.1: Age at diagnosis of tuberous sclerosis complex (n=382)



Symptoms present at the time of diagnosis of tuberous sclerosis complex

The symptoms of TSC present at the time of diagnosis (n=1231) could be compared to all symptoms present in our group of 370 patients (n=2728), i.e. 45%, with no difference between major symptoms, minor symptoms or incidental findings. Often present at the time of diagnosis and thus contributing to making the diagnosis were epilepsy (infantile seizures

especially), cardiac rhabdomyomas and pulmonary lymphangioleiomyomatosis (LAM). LAM, although rare in itself, is an important clue to the diagnosis of TSC in some patients. The 'classical' signs like mental retardation, facial angiofibroma, cortical tubers or subependymal nodules showed percentages close to the overall average.

Our data indicate that the clinical assessment at the time of diagnosis is incomplete and, that 55% of symptoms are found either after more detailed investigation following the diagnosis of TSC, or develop later in life. Complete assessment and regular follow-up of TSC patients will lead to higher and more accurate estimates of point as well as life time prevalences of signs and symptoms. A protocol for follow-up investigations has been published by Roach et al.(1999), and will be discussed in Chapter 4. It is likely that on average the ages presented in the following sections are often higher than the age of onset of the symptom, due to different kinds of reporting delays. The data are a rough estimation however, of what percentages of symptoms are at least present in a large group of patients, and the relative differences between symptoms within a group.

Skin signs

The most obvious and rather distinctive skin sign of TSC in the registry is facial angiofibroma, which was scored 306 times (263 times present, 43 absent). Hypomelanotic macules were reported for 280 patients, and in a minority of cases sub-specified according to shape. In total, one or more positive skin signs were reported in 359 patients (Table 2.1). When a detailed dermatological and/or clinical genetic report focused on the detection of TSC related symptoms mentioned 'no skin abnormalities', this was interpreted as absence of facial angiofibroma, hypomelanotic patches, and ungual fibromas. The male/female ratio of the fibrous forehead plaques of 1.43 is unexplained and should be expored in further studies.

The frequencies reported in this table provide an estimate of the prevalence of each symptom in the total study population (n=370). The lowest estimate of the prevalence is obtained by assuming that for patients not informative for a specific symptom that particular symptom was absent. The highest estimate is obtained by assuming that the uninformative patients were affected for that symptom. For facial angiofibroma, this results in a lower estimate of 263/370 (71%) and a higher estimate of 327/370 (88%) patients. For white macules this range is 73%-95%, for ungual fibromas 30%-78%. Gomez reported a frequency of 83% for angiofibromas, 89% for white patches, and 52-88% for ungual fibromas in adults (Gomez 1999). One should be aware that the data from most publications, like this study, are based on data obtained in studies with mixed cross-sectional and longitudinal elements in their design.

Table 2.1: Skin signs in 359 TSC patients

Skin sign	No. Patients Informative	No. Symptom Absent	No. Symptom Present (%)	M/F ratio
Facial angiofibroma	306	43	263 (86)	1.04
Hypomelanotic macules, unspecified	280	19	261 (93)	1.02
Hypomelanotic macules, ash leave			48	
Hypomelanotic macules, confetti			13	
Hypomelanotic macules, fingerprint			11	
(Sub)ungual fibromas	191	80	111 (58)	1.06
Shagreen patch	171	58	113 (66)	1.06
Fibrous forehead plaque	104	72	32 (31)	1.43
Other skin signs:				
Cafe-au-lait spots			26	
Molluscum fibrosum pendulum			24	
Poliosis/absent hair pigment			6	
Synophrys (fused eyebrows)			2	
Hypertrichosis (hair overgrowth)			2	
Malignant melanoma			1	

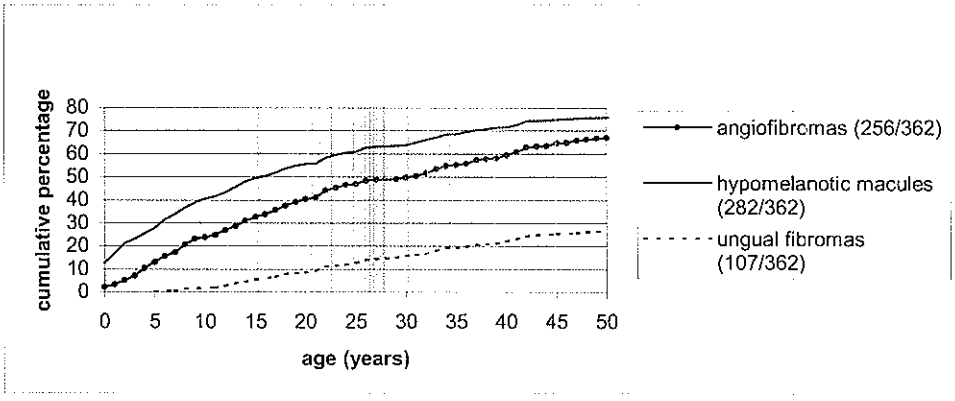
Age of onset of skin signs: angiofibroma, ungual fibroma and white macules

Frequently, hypomelanotic macules (white spots) were the first skin sign of TSC, usually either present at birth or appearing in the first year of life. Age of documentation tertiles for hypomelanotic macules were therefore close to those of age of diagnosis, as could be expected for a stationary sign that is relatively easy to document.

Facial angiofibroma can be congenital, but usually appears in the first years of life, typically between the second and fifth year. The number of patients with angiofibroma increases around puberty, whereas appearance after the age of twenty years seems rare (Gomez 1988). In our registry, the diagnosis of facial angiofibroma was reported by the age of 20 years in about two thirds of all persons who were eventually diagnosed with angiofibroma (Figure 2.2). After 20 years, the shape of the curve follows closely that of the diagnosis curve. These patients had probably noticed the facial papules earlier, but they were not aware that they had TSC. The rise after 20 years of age could also mean that at higher age the angiofibroma is an important sign for the diagnosis of TSC. The group of patients diagnosed as a result of cascade screening following the identification of younger index patients make up a large proportion of this number.

Ungual fibromas were found in only around 30% of all patients, and were either noted late or developed later in life. Ungual fibromas usually arose after the age of 5 years and tend to be reported more regularly in the older age group. Figure 2.2 compares the cumulative incidence curves for the three major skin signs above.

Figure 2.2:Age at documentation of skin signs (n=362)



Structural central nervous system abnormalities

Central nervous system abnormalities can be divided in functional and structural abnormalities. Cranial imaging is generally not part of the medical work-up in standard epilepsy protocols but is done when the epilepsy is not the only sign (of TSC) present in a child. The contribution of CT-scan or MRI scan results to the actual diagnosis of TSC was limited in our dataset and this investigation rather appeared to be confirmatory for the diagnosis. CNS abnormalities were often documented with a substantial delay, especially in the 'older' TSC patient. Not all patients underwent cranial imaging, especially those with a 'clear cut' diagnosis, who would not be able to hold still during a scanning procedure, because of young age or restlessness. Structural brain manifestations were reported in a group of 326 patients i.e. 88% (Table 2.2), compared to about 90% in the literature (Tuberous Sclerosis Complex 1999).

Subependymal nodules (SEN) and (sub)cortical tubers are the predominant signs of the brain. While conventionally CT scan is considered superior to MRI scan to detect calcified as well as non-calcified SEN, cortical tubers are better seen on the MRI scan, because of its more accurate definition of white and grey matter. Since the MRI scan has been introduced in medical practice much later than the CT scan and is less convenient for the examination of very young patients, as sedation is needed, cortical tubers and dysplasias may be underreported in our series that covered the period between 1988 and 1999. In the recent literature (Tuberous Sclerosis Complex 1999), a prevalence of 80% is given for SEN's and

90% for (sub)cortical tubers. Since the population studied by Gomez is largely symptomatic, the high frequency of (sub)cortical tubers might represent a slight overestimation.

Table 2.2: Brain abnormalities in 326 TSC patients

Brain abnormality	No. Patients Informative	No. Symptom Absent	No. Symptom Present(%)	M/F ratio
Subependymal nodules (SEN)	200	12	188(94)	1.09
(Sub)cortical tubers	115	20	95(83)	1.24
Ventricular dilatation			29	
CT abnormality, unspecified	54	26	28	
Brain calcification			47	
Subcortical hypodensities			21	
Subependymal giant cell astrocytoma			19(5.1)	1.00
MRI abnormality, unspecified			9	
Brain atrophy			5	
Brain hamartoma			3	
Hydrocephaly			3	
Megalencephaly			2	
Structural brain defect, unspecified			1	
Cerebellar hypoplasia			1	
White matter defect			1	
Ependymoma			1	
Brain tumour, unspecified			1	
Ventricle defect			1	

Subependymal giant cell astrocytoma (or SEGA) is a complication of the subependymal nodules, and was reported in the literature in 6.1% of the patients (Tuberous Sclerosis Complex 1999). We recorded 19 cases (5.1%), with an age at diagnosis between birth and fifty years. Most cases occurred between 5 and 15 years of age, but six of the nineteen cases were reported after the age 20 years, causing death in at least two of these cases and probably in a third case. This is a remarkable result and has consequences for screening at adult age in patients with centrally localised lesions. In most of the existing literature, SEGA's are considered not to appear after the age of 20 years. Our observations indicate that late ascertainment of SEGA's is associated with a higher rate of fatal complications

Eyes

TSC of the eye manifests mostly as retinal hamartomas and depigmentations. The hamartomas are mostly located in the periphery of the retina and may easily remain undetected during routine eye examination not focused on the detection of TSC-related signs. Symptoms of the eye were recorded present in 81 patients (22%), less than the percentage reported in the literature, as many patients did not undergo an ophthalmological assessment. In this group, 46 patients had a retinal hamartoma (57%). Retinal hamartoma was excluded in 143 of the 370 patients (39%). All eye symptoms recorded are presented in the Table 2.3. Again we found a preponderance of male patients in this group, as was also obseved for the fibrous forehead plaques of the skin. An obvious explanation for this difference may be that more male patients were investigated than females, but this data is lacking in our dataset as for many patients only abnormalities were reported.

Table 2.3: Eye abnormalities in 81 TSC patients

	No of patients	M/F ratio
Retinal phakoma	46	1.73
Retinal pigmentation abnormality	21	1.81
Strabismus	10	
Other retinal abnormality	4	
Cataract	4	
Nystagmus	3	
Iris coloboma	3	
Hypertelorism	2	
Optic nerve atrophy	2	
Anisocoria	1	
Coloboma of the eyelid	1	
Iris pigmentation defect	1	
Iris heterochromia	1	
Lens coloboma	1	
Optic nerve abnormality unspecified	1	
Papilledema	1	
Pupil position defect	1	

Like the hypomelanotic macules of the skin, the retinal abnormalities are a stationary sign. Two thirds of the patients eventually known with retinal hamartoma were less than 20 years at the time of documentation of the eye symptom, in line with the overall proportion of patients diagnosed with TSC at age 20 years. The eyes, especially the optic nerve and retina, are often

regarded as an extension of the central nervous system (CNS). The possibility of a correlation between ophthalmologic findings and CNS abnormalities in TSC patients was investigated and data are given in Table 2.4.
Between the groups with and without eye abnormalities, no differences were observed with respect to the proportion of patients with CNS abnormalities. The table shows that when the eyes have not been investigated, a lower proportion of persons has CNS abnormalities than in the group that did have eye examinations. A possible explanation might be that neurological signs or investigations lead to a visit to the neurologist who performs ophthalmologic examination or refers for it, whereas in the absence of neurological signs, the ophthalmologic examination may be skipped more frequently.

Table 2.4: Eye abnormalities versus brain abnormalities in 370 TSC patients

Eye signs	CNS abnormalities Present	CNS abnormalities Absent/unknown	Total	%*
Eye sign(s) present	49	11	60	82
Eye sign(s) absent	118	27	145	81
Unknown	107	58	165	65
Total	274	96	370	74

%= CNS abnbormalities present/total number of patients investigated

Functional central nervous system abnormalities

The major functional problems in TSC patients are epilepsy and mental retardation. Epilepsy is a frequent symptom of TSC, and was present in 74% of the registered TSC patients. When the epilepsy occurred early in life, especially as infantile spasms (salaam type), there was a high proportion of mental impairment: severe mental retardation, behavioural problems and autism-like symptoms. Mental retardation was diagnosed in just over 54% of the patients; severe mental retardation was only seen after the occurrence of epilepsy, with the exception of a few cases described below, in whom the mental retardation could be ascribed to other causes. In most cases, the mental retardation was not diagnosed in the first two years of life. Some late ages of registration of mental retardation were due to incomplete data about the exact age of diagnosis of the mental retardation, in which case the age at reporting was recorded.
In our study population of 370 patients, 275 (74%) were reported with seizures at any period during their lifetime. Of these 275 patients, 64 patients (23%) had infantile spasms. In 56% (36/64) of the patients with infantile spasms, subsequently other seizure types were reported. This will probably not reflect the normal course of seizure development, because of limited

follow-up information on most of these patients. An overview of the different types of seizures is given in Table 2.5. There was a relatively large group of unspecified seizures (n=148), mainly due to lack of detailed seizure history.

Table 2.5: Reported epileptic seizures in 275 TSC patients

Seizure type	Number of patients	Frequency (%)
Generalised seizures		
Absence seizures, atypical	4	1
Absence seizures, myoclonic	1	<1
Absence seizures, unspecified	29	11
Myoclonic	5	2
Clonic	3	1
Tonic	10	4
Tonic-clonic	24	9
Atonic	4	1
Unspecified	12	4
Partial seizures		
Simple partial	10	4
Complex partial	33	12
Secondarily generalised	15	5
Unspecified	10	4
Infantile spasms	64	23
Neonatal seizures	3	1
Seizures during fever	14	5
Status epilepticus	7	3
Seizures, type unspecified	148	54
TOTAL	396	

Original reports of EEG examinations were available in a minority of patients with epilepsy (n=60) and described as 'epileptic' in 35, 'hypsarrhythmia' in 10, 'paroxysmal discharge' in four, background abnormality in two, and normal in nine.

In 83 epileptic patients (30%), more than one type of epilepsy was recorded (Table 2.6), although not necessarily at the same period during their life. In just over half of the 64 patients with infantile spasms the seizures had developed into another type of epilepsy,

sometimes after a seizure-free interval. In about 10% of patients three or more different types of seizures had occurred during or before the period during which the current information was obtained.

Table 2.6: Concurrence of different types of epileptic seizures in 275 TSC patients

Number of seizure types in a patient	Number of patients
1	192
2	56
3	20
4	3
5	4

Epilepsy and mental retardation

It is generally accepted that in TSC severe mental retardation does not occur without pre-existing epilepsy, with onset most often in the first year of life. Of the total group of 370 TSC patients, 199 (54%) were reported to have some degree of mental retardation, either mild (65 patients, M/F=0.84), moderate to severe (103, M/F=1.23), or unspecified (31, M/F=1.5). Formal and comparable IQ test results were not available for our study. The degree of the mental retardation was recorded from the medical notes or entry forms.

Among the patients with epilepsy, mental retardation was present in 70% (192/275). In the subgroup of patients with infantile spasms 88% (56/64) had mental retardation. Of these 56 patients, 43 (77%) had severe mental retardation, 8 (14%) had mild retardation, and for 5 patients (9%) the degree of retardation was not specified. For some patients aphasia or delayed speech was reported separately. Table 2.7 summarises the comparison of mental retardation and epilepsy.

Table 2.7: Epilepsy versus mental retardation in 370 TSC patients

	Epilepsy present	Epilepsy absent	Total
Mild mental retardation	59	6	65
Severe mental retardation	102	1	103
Mental retardation, unknown severity	31	0	31
No mental retardation	83	88	171
Total	275	95	370

Mental retardation seemingly without epilepsy, occurred in seven patients: six times borderline or mild retardation, once severe retardation. Our data support earlier observations (e.g. Gomez, 1999) that in TSC patients mental retardation only occurs mainly in patients who have epileptic seizures and is not part of the clinical spectrum of TSC when epilepsy is absent.

In view of the close connection between the occurrence of epileptic seizure and mental retardation, it is worthwhile to summarise the clinical details of those patients who were mentally retarded but did not have any seizure history.

Patient 1: A boy, 7 years old, with mild mental retardation and cortical tubers on the MRI-scan of the brain, had no previous history of epilepsy. EEG data were not available for this patient.

Patient 2: This girl, 11 years old, had moderate mental retardation. She was one of a pair of twins born after 7½ months of pregnancy and needed six weeks of intensive neonatal care. The other twin died two days after birth because of hyaline membrane disease. In the first months of her life, development appeared more delayed than would be expected as a result of her premature birth. The CT scan of her brain showed enlarged ventricles and brain asymmetry. EEG examinations at the age of 2 years and 9 months, and 4 years and 4 months were normal.

Patient 3: This 14 years old borderline retarded boy had mainly memory problems. He had angiofibroma and white spots. The earlier CT scan of his brain showed subependymal nodules and hydrocephalus. Epileptic seizures had never been observed. His EEG showed focal discharges of the left temporo-occipital horn, but no definite epileptic activity. After surgical removal of a giant cell astrocytoma, the postoperative EEG showed a normal pattern. He had experienced unexplained precocious puberty at 10 years of age.

Patient 4: This 24 years old woman had angiofibroma and white macules. Her MRI scan showed cortical tubers and an astrocytoma, which was operated. Her retardation was reported as borderline. Her mother was also affected with TSC. No further data were available.

Patient 5: This 41 years old Polish mother of a severely retarded epileptic TSC patient had herself also a 'mild degree of mental retardation'. Her past medical history was largely unknown, but epilepsy had not been reported. EEG data for this patient were not available.

Patient 6: This 42 years old male patient had multiple dysmorphic features: hypoplastic malae, downward slant of the eyes, dysplastic ears, high nasal bridge, micrognathia, triphalangeal thumbs and bilateral hearing loss. He had been diagnosed with a severe mental retardation from shortly after birth. His features of TSC were facial angiofibroma, shagreen patch and ungual fibroma. At the age of 44 years polycystic kidneys were diagnosed. He had no known history of epilepsy and no data on EEG's were available. His deep mental retardation was more likely syndromal than to be connected to his TSC. In the differential diagnosis Nager syndrome, (mandibulofacial dysostosis [MIM154400])

and Townes Brocks syndrome [MIM107480] (anal abnormalities are not obligatory in this syndrome) had been considered but could not be proven with certainty. Detailed high-resolution chromosomal investigations were normal. Because of a possible localisation of Nager syndrome near the telomere of chromosome 9q, FISH studies were done using chromosome 9 probes, from the TSC1 region (9q34), but showed no abnormalities. Mutation analysis of the TSC2 gene resulted in the identification of a large deletion, encompassing both the complete TSC2 gene and the adjacent PKD1 gene.

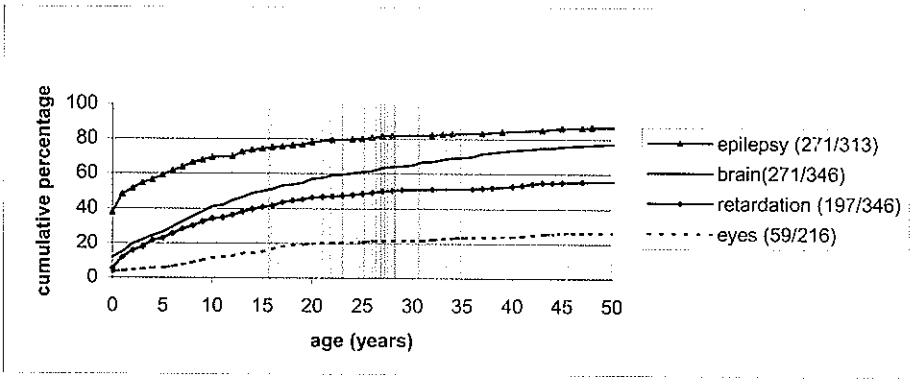
Patient 7: This half of a monozygotic, concordantly affected twin sister pair was mildly retarded. She had no seizures recorded in her medical files or noticed by the mother. On retrospect, at birth their mother had noticed facial angiofibroma in both twins. At fifteen months of age the second sister was diagnosed with an unexplained hemiplegia and hemiatrophy of the brain. She was then noticed to have white macules. She was equally mildly retarded as her sister, and had epileptic seizures and a 'borderline epileptic activity' EEG. On CT scan of the brain paraventricular nodules were seen in both sisters, with cortical tubers in the 'epileptic' sister and none in the other sister.

These seven medical histories do not provide a specific clue with respect to the question why the patients were mildly retarded despite the absence of manifest epilepsy. In one case, subclinical epileptic EEG signs had disappeared after neurosurgical removal of a giant cell astrocytoma, and in one case epilepsy was absent despite the presence of cortical tubers and an astrocytoma. On the other hand the monozygotic twin pair (case number 7) showed parallelled discordance for epilepsy as well as cortical tubers.

Age of documentation of central nervous system abnormalities

In Figure 2.3 the ages of documentation of the central nerve system abnormalities show presence of epilepsy in the first year of 40%-50%. In our registration over time the percentage of structural brain abnormalities 'catches up' with the percentage of epilepsy. This is not a true reflection of the order of events in TSC, but an artefact due to several reasons. Epilepsy is a rather easy sign to diagnose and can be apparent before the underlying brain abnormalities are detectable. Secondly, the detection of brain abnormalities requires imaging of the brain. The development of the neurocranial imaging techniques over the last 30 year has been remarkable. In the early developmental stages of CT and MRI scanning technology, older patients could undergo imaging, whereas young patients could not because of problems with sedation or anaesthesia.

Figure 2.3: Age at documentation of CNS signs in TSC (n=346)



Cardiovascular signs

Most data on the cardiac abnormalities were obtained from cardiac ultrasound investigations, often around the time of diagnosis, or in the process of genetic work up for genetic counselling (Table 2.8). As the clinical value of cardiac ultrasound in TSC is limited after the age of one year, this investigation has not been done or recorded consistently. A normal cardiac ultrasound result was given for 72 patients. In these cases, cardiac tumour and structural heart defect categories could be scored absent.

Table 2.8: Cardiac abnormalities in 73 TSC patients

	No of patients	M/F ratio
Cardiac tumour		
Rhabdomyoma	35	0.94
Tumour unspecified	11	
Cardiomegaly	8	
Dysrhythmia/ECG abnormality	17	
Murmur unspecified	8	
Systolic murmur	5	
Congestive heart failure	3	
Increased blood pressure	2	
Cardiovascular shock	2	
Cor pulmonale	1	
Structural heart defect unspecified	1	
Mitral valve stenosis	1	
Mitral valve incompetence	1	

Aorta defect	1
Aorta dissection	1
Brain artery defect	1
Varicose veins	1
Arteriovenous malformation	1

Kidneys

Renal abnormalities are not only relatively frequent symptoms of TSC but also important targets for early intervention in order to prevent the development of renal insufficiency. This is especially true for large angiomyolipomas (AMLs). Regular ultrasound investigations are recommended in TSC patients with renal abnormalities, as angiomyolipomas tend to grow. Surgeons and urologists often advise preventive embolisation of angiomyolipomas measuring over 3.5-4 cm in diameter, because of a substantially increased risk of haemorrhage. In the TSC registry, no systematic follow-up data were collected, but some follow-up data is presented in Chapter 3.4. In the clinical registry form of 104 patients the statement 'no renal cysts' was given and in the form of 90 patients 'no structural renal defect' was reported. Table 2.9 shows the available data. For AMLs and renal cysts a male/female ratio of 0.7-0.8 was seen., not surprising as it is known from the literature that a higher proportion of women develop AMLs and on average have larger AMLs (Gomez 1999).

Table 2.9: Reported renal abnormalities in 108 TSC patients

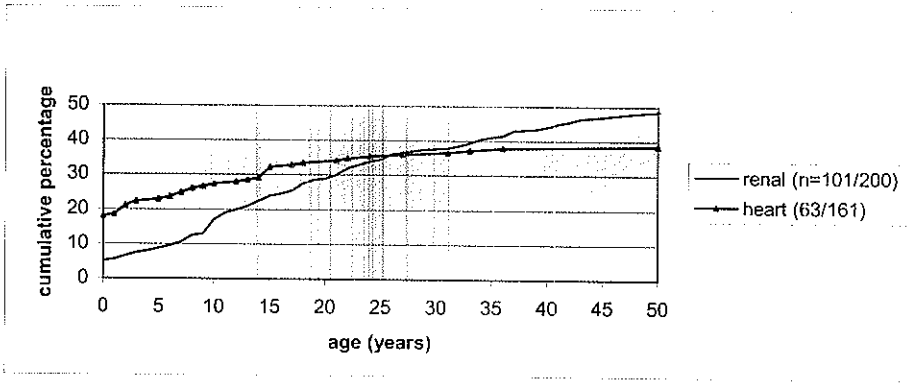
Renal abnormalities	No of patients	M/F
Angiomyolipomas	67	0.66
Multiple renal cysts	39	0.77
Size shape or structural defect	13	
Haematuria	8	
Flank pain	5	
Renal cell carcinoma	4	
Palpable flank masses	3	
Renal insufficiency	3	
Urinary tract bleeding	2	
Absent kidney, unilateral	1	
Single renal cyst	1	

Age at documentation of heart and kidney abnormalities

Rhabdomyoma, the characteristic heart abnormality in TSC patients, is congenital and can be diagnosed during pregnancy, at birth or in the first year of life. Smaller rhabdomyomas can be completely asymptomatic and are only picked up when a person is screened for cardiac abnormalities. The normal clinical course of rhabdomyoma is regression with age. Therefore when a diagnosis of TSC is made late in life, the chance is comparatively small that cardiac abnormalities are detected. The slope of the curve of the heart abnormalities is therefore less than the age at diagnosis curve in Figure 2.1. From 161 individuals the result of the cardiac examination was known, of whom 39% had an abnormality (i.e. 17% of the whole group of 370). Cardiac problems like conduction disorders may sometimes become manifest later in life.

Renal abnormalities can be present as congenital multiple renal cysts or sometimes as polycystic kidney disease. The prevalence of renal symptoms in the patients in our study was 29%; just over half of the patients had their symptoms before twenty years of age. Compared to the previously reported percentages, this prevalence was low, which is probably largely due to incompleteness of investigation of our study population and lack of systematic follow-up data for the whole group (Figure 2.4).

Figure 2.4: Age at registration of heart and kidney abnormalities



Lungs

Lung manifestations are reported for a small number of patients. From the literature it is known that the lung is almost exclusively affected with TSC in female patients. All patients with lung symptoms were female, except for one male patient who had a pneumothorax (Table 2.10)

Table 2.10: Lung abnormalities in 11 TSC patients (10 female, 1 male patients)

	No patients
Pneumothorax	6
LAM	2
Respiratory distress	2
Lung cysts	1
Apnoea	1
Emphysema	1

Dentition/oro-nasopharynx

Data on enamel pitting was available for 91 patients. In 23 patients no enamel pits were present. Other intra-oral symptoms were seen in 28 patients (Table 2.11).

Table 2.11: Abnormalities in teeth, gingiva or other structures in the mouth in 88 TSC patients

	No patients
Enamel pits	68
Gingival fibromas	23
Hypertrophied gingiva (phenytoin-associated)	2
Frenulum defect	1
Gingiva defect, unspecified	1
Oropharyngeal fibroma	1

Other signs and symptoms reported

Other abnormalities in the group of 370 TSC patients are summarised in table 2.12. Many of these symptoms have only been recorded once, and might represent co-incidental findings. Some others, like hemihypertrophy and (osteo)sclerosis of the bones, may be part of the TSC spectrum.

Table 2.12: Other clinical features reported in 370 patients with a diagnosis of TSC

Sign	No reported
Hemihypertrophy	4
Facial abnormalities	
Epicanthal folds	2
Facial haemangioma	2
Nevus flammeus of eye lid	2
Ear defects	
Hearing loss	5
Microtia	1
Other ear shape defect	1
Skull abnormalities	
Frontal bossing	1
High frontal hair line	1
Low posterior hair line	1
Bone and joint abnormalities	
Sclerosis	12
Scoliosis	9
Increased ossification of the skull	7
Macrocephaly	4
Pseudocysts	4
Club foot	2
Flat foot	2
Increased density of bone	2
Retarded bone age	2
Vertebral listhesis	2
Clinodactyly	1
Coxa valga	1
Decreased range of motion of joints	1
Hip joint defect	1
Hip dislocation	1
Hip dysplasia	1
Limb defect	1
Lordosis	1

Lower limb asymmetry	1
Malignant osteosarcoma	1
Osteoma	1
Pectus excavatum	1
Pes cavus	1
Radius defect	1
Sandal gap	1
Syndactyly of toes	1
Triphalangeal thumbs	1
Neuromuscular defects	
Hypotonia	11
Muscle paresis	6
Hemiplegia	5
Inguinal hernia	2
Paraplegia	2
Spasticity	2
Diaphragm defect	1
Diastasis recti	1
Other neurological manifestations	
Neurological defect unspecified	4
Hyperreflexia	2
Ataxia	1
Cerebral palsy	1
Encephalitis	1
Facial nerve defect	1
Truncal ataxia	1
Behavioural problems	
Aggressive behaviour	8
Autism	7
Behavioural defect unspecified	7
Temper outburst	3
Apathy/depression	2
Self destructive behaviour	2
Sleep disorder	1
Stereotypy	1

Gastrointestinal manifestations	
Liver angiomyolipoma	13
Liver cysts	2
Polyps colon/rectum	2
Pancreas adenoma	2
Hepatomegaly	1
Malignant pancreatic tumour,	1
(Uro)genital/hormonal abnormalities:	
Early puberty	2
Bladder carcinoma	1
Cryptorchidism	1
Hypothyroidism	1
Thyroid gland defect	1
Uterus abnormality	1
Haematopoietic system	
Anaemia	1
Granulocyte defect	1
Immunoglobulin defect	1

Causes of death in patients with tuberous sclerosis complex

Death was reported in 23 patients (M/F = 9/13, 1 unknown gender). The average age at death for these male patients was 23.6 years (range 0-65y) and for the female patients 22.1 years (range 0-54y). The patients are described in ascending order of age at death (Table 2.13 and Figure 2.5).

Patient 1: This foetus of unknown gender was diagnosed with intrauterine death in the first pregnancy of a mother with TSC. The baby was affected with severe cardiac rhabdomyoma. No further information is available about this case.

Patient 2: This baby girl died one day after birth from congenital cardiac rhabdomyomata. She had subependymal nodules and cortical tubers of the brain and renal cysts.

Patient 3: A baby boy died 5 days after birth because of complications of his congenital cardiac rhabdomyomas. Post mortem examination was done including brain pathology, which confirmed the diagnosis tuberous sclerosis complex of the brain. No further details were available on this patient.

Patient 4: Death due to congestive heart failure with Wolff Parkinson White (WPW)

syndrome occurred in a one-month old girl with inoperable congenital cardiac rhabdomyomas and cortical tubers in the brain.

Patient 5: This boy was one of probably dizygotic male twins that were discordant for TSC. He had cardiac arrhythmias due to rhabdomyomas with WPW syndrome, infantile seizures and unilateral eyelid coloboma. On a brain CT scan periventricular calcifications were observed. He developed sepsis, possibly related to ACTH treatment for his seizures, and died subsequently at the age of 8 months.

Patient 6: A 10 months old boy, with severe right-sided epileptic seizures with hypsarrhythmia, mental retardation and hypomelanotic macules. CT scan showed multiple paraventricular nodules. He died of unknown cause.

Patient 7: The patient was a 15-year-old boy, whose cause of death was massive bleeding following brain surgery for a giant cell astrocytoma. He had no evident epilepsy or mental retardation. The diagnosis of TSC was made only several years after his death, when his father was diagnosed with facial angiofibromas. This symptom was also present in the boy. The kidneys, heart and liver had been used for transplantation, and had been without signs of TSC. Eurotransplant was informed about this retrospective diagnosis. Follow-up information on the fate of these organs was not available for this study.

Patient 8: A cardiovascular shock occurred in this 16 years old female patient. She probably died of gastrointestinal bleeding of which the exact source has remained unknown. She had epilepsy, was severely mentally retarded, had a brain tumour (probably giant cell astrocytoma) and renal angiomyolipoma.

Patient 9: This 16 years old girl died unexpectedly, probably from a sudden pneumonia. She was a patient with severe mental retardation, epilepsy, cystic kidneys and had had unexplained recurrent episodes of fever in the period before her death.

Patient 10: In this female patient aged 17 years, death followed renal bleeding from an angiomyolipoma. She was severely retarded with epilepsy and had pre-existent flank pain and unexplained periods of fever and haematuria.

Patient 11: This 17 years old boy developed increased intracranial pressure that could be reduced successfully twice. Nevertheless, he went into coma and died 6 weeks later. He was known with hypotonia following vaccination for DTPP (diphtheria, tetanus, poliomyelitis, and pertusis) vaccination at 5 months of age. He had severe epilepsy, mental retardation, spasticity, facial angiofibroma and white maculae on the skin.

Patient 12: An 18 years old female died from metastases in the lungs and in the cerebrum. The primary tumour she had was a malignant osteosarcoma, growing in the left distal femur, which had been diagnosed three years earlier. She was a patient of normal intelligence with facial angiofibroma, but otherwise with few clinical signs of TSC.

Patient 13: At 20 years of age this female patient died from metastases of malignant melanoma of the skin. She had epilepsy, mental retardation and renal angiomyolipomas. She also had heart rhythm disturbances from rhabdomyomas.

Patient 14: This 22 or 23 years old male patient, only known from information provided by

the family, probably died of an epileptic seizure. He was known to have had epileptic seizures, facial angiofibroma and white macules.

Patient 15: This 23 years old female patient was found dead at home. She most probably died from an epileptic seizure. She was from a large TSC family and had facial angiofibroma. No post mortem examination was performed.

Patient 16: A 25 years old male patient was known with epilepsy, mental retardation and a symptomatic giant cell astrocytoma. After brain surgery in order to remove his brain tumour, he had severe haemorrhages that were fatal.

Patient 17: This 39 years old female patient died from respiratory insufficiency due to lymphangioleiomyomatosis of the lungs. She had been on oxygen therapy for the last one and a half years before her death. Other manifestations of TSC in this patient were giant cell astrocytoma, cortical tubers and renal angiomyolipoma.

Patient 18: A female patient, who was severely affected with epilepsy and mental retardation died at 40 years. She had her right kidney removed some years earlier because of angiomyolipomas. The probable cause of death was cardiovascular shock because of bleeding from the left kidney, which was similarly affected with large angiomyolipomas.

Patient 19: A 40 years old male TSC patient from a TSC family, who had facial angiofibroma, died from pulmonary embolism.

Patient 20: A 44 years old female patient, mother of patient 19 had died from bleeding from a renal tumour. She had been known with facial angiofibroma.

Patient 21: This male, 50 years of age, died from pneumonia. He had been surgically treated for a subependymal giant cell astrocytoma previously. Post mortem investigations of the brain showed the presence of subependymal nodules and cortical tubers.

Patient 22: This 54 years old female patient had many manifestations of TSC, including facial angiofibroma, nail fibromas, hypopigmented spots on her back and on her abdomen, calcified subependymal nodules and subcortical tubers, She had undergone left-sided nephrectomy at 37 years of age because of angiomyolipoma. In the right kidney she had developed a renal cell carcinoma, with metastases to the lung. She had liver hamartoma and severe pulmonary lymphangioleiomyomatosis, with pulmonary emphysema, leading to pulmonary hypertensive heart disease and eventually heart failures.

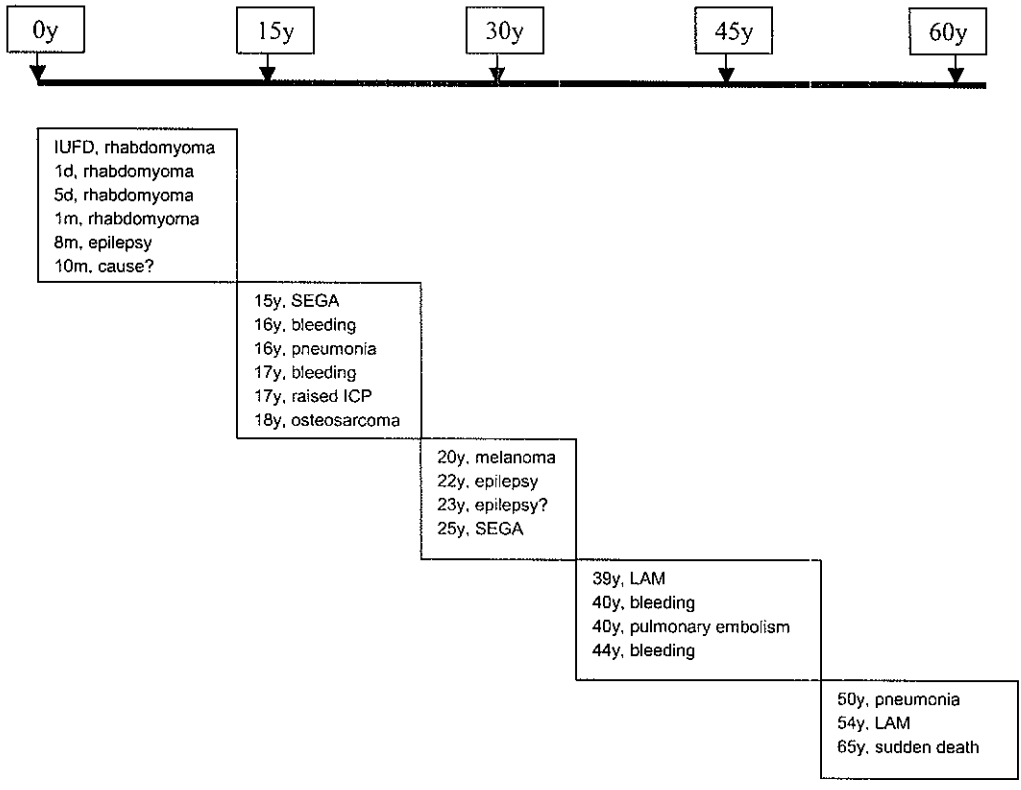
Patient 23: Sudden unexplained death occurred in this patient at 65 years of age, following complaints of dizziness some months before, which had been ascribed to insufficiency of the cerebral vessels. He was the grandfather in a three-generation family with TSC. Permission for a post mortem investigation was not obtained. He was a man without retardation or epilepsy, with quite severe periungual fibromas. The fibromas had been explained to him to be the consequence of his job in a mustard factory! His previous medical history reported myocardial infarct, and an operation for a spinal hernia.

Table 2.13: Reported causes of death in 23 TSC complex patients

Organ system	Supposed cause of death
Central nervous system (6x)	Epilepsy (3x) Subependymal giant cell astrocytoma (2x) Raised intracranial pressure
Cardiovascular (8x)	Rhabdomyoma (4x) Bleeding (4x)
Respiratory system (5x)	Lymphangioleiomyomatosis (2x) Pneumonia (2x) Lung abscess
Other (4x)	Malignancies* (2x) Unknown cause (2x)

* Two TSC unrelated tumours: metastasised melanoma and osteosarcoma of the femur.

Figure 2.5: Schematic representation of reported causes of death in 23 tuberous sclerosis patients, on a time axis from 0 years to 60 years



Abbreviations: IUFD = intra-uterine foetal death, SEGA = subependymal giant cell astrocytoma, ICP = intracranial pressure, LAM = lymphangioleiomyomatosis of the lungs. d = day, m = month(s), y = year(s)

Conclusions:

The data collected in our study indicate that a wide range of signs and symptoms can be found in TSC. It is clear that the spectrum of clinical manifestations displayed by one patient is dependent on his or her age. Therefore, the age at clinical assessment of the patient is of critical importance. As the assessment age is largely determined by the age at diagnosis of TSC, any study of prevalences and ages at onset of individual symptoms is difficult to standardise. Some of the limitations of our study when trying to gather at least an impression of prevalences and ages are presented in Chapter 2.1.

Our data on patients with mental retardation without epileptic seizures show that in all cases an alternative explanation for the retardation could be given. Thus, the hypothesis that in TSC mental retardation only occurs in patients with seizures is confirmed. It is unknown whether there is a causal relationship between epilepsy and mental retardation, or if there is a common underlying cause at the time of brain development and neuronal migration.

The mortality review shows, that in the large majority of patients who died young, a relationship was evident with disease features of TSC, such as cardiac failure, epilepsy, renal haemorrhage, brain astrocytoma or pulmonary lymphangioleiomyomatosis. In Chapter 4 diagnostic protocols and follow-up schedules are provided, aimed at timely intervention and further improvement of our insight in the natural course of TSC. Extrapolation of the data could aid in identifying more precisely the health risks for individual TSC patients and the needs for preventive strategies.

In order to set up a proper epidemiological study of the prevalence of symptoms in TSC and to construct proper age of onset curves, a group of (initially young) patients should be followed up in a special clinic, with regular times of assessment according to a standardised protocol. Sporadic patients will be diagnosed only after the manifestation of one or more clinical problems like epilepsy, developmental delay or skin problems. Therefore the early development of subtle signs has to be studied in children from TSC families, while taking into account that the familial nature of the disease implicates at least partly preserved reproductive fitness possibly reflecting relatively milder mutations or phenotypes in this group of patients. In families with a known mutation, gene mutation carriers can be followed up from birth (or before birth even), in order to determine the natural course of TSC at young age. Close monitoring, especially for beginning signs of infantile spasms, which often start with subtle absences (staring gaze) and early treatment with anti-epileptic drugs is thought to have a favourable effect on seizure control and potentially influence the chance of mental retardation. In Chapter 4, a follow-up schedule is given published by Roach et al (Roach et al. 1999) as a consensus scheme for clinical purposes, focussing on relevant monitoring from the point of view of the patient. For better insight into the natural course of TSC, the schedule in Chapter 4.1 should be extended into a protocol, which can be of use in the setting of a specialised TSC follow-up clinic.

2.2.3 TSC1 versus TSC2 phenotypes

In Chapter 3.2, data are presented on the spectrum of mutations in the group of patients with a TSC1 mutation (van Slegtenhorst et al. 1999) and the percentages of clinical manifestations of these patients are given. No evidence was obtained that a subgroup of TSC patients with a TSC1 mutation is phenotypically distinct from the average TSC population. In several other publications a genotype-phenotype correlation between TSC1 and TSC2-related disease was suggested (Jones et al. 1999; Dabora et al. 2001; Jones et al. 1997). In a group of 150 unrelated patients, Jones reported significantly more frequent mental disability for patients with a TSC2 mutation compared to those with a TSC1 mutation. The study of Dabora et al. supports the findings of Jones et al. and report lower frequencies for renal cysts, angiomyoliopomas, forehead plaques, retinal hamartomas, and milder angiofibroma for TSC1-related disease (Dabora et al. 2001)

Genotype-versus-phenotype studies concerning TSC are complex. Causes of bias may be encountered, corrections for which create other biases. Firstly, the proportion of families linked to either the TSC1 gene or the TSC2 gene is roughly equal. For sporadic patients, however, evidence until now points at a large majority being due to a TSC2 mutation. The total number of sporadic patients with a TSC1 mutation is still small, therefore a good comparison of sporadic patients with a TSC1 and those with a TSC2 mutation has not been possible yet. Studies without recruitment bias are lacking. Most of the patient series were selected for gene identification studies, and consisted initially of familial cases rather than sporadic cases. Since a severe phenotype obviously interferes with reproductive fitness, we may expect that sporadic patients have on average a more severe phenotype than familial cases. As sporadic patients are found to have a TSC2 mutation more often than a TSC1 mutation, it thus seems logical that the average 'TSC2 phenotype' in the populations studied so far are more severe than the 'TSC1 phenotype'. Our data on all patients with a known mutation support those of Jones et al. and Dabora et al, probably because of the similar biases (Table 2.15). Jones et al. reported a significantly higher frequency of mental impairment for patients with a TSC2 mutation compared to patients with a TSC1 mutation. Our data supported this slightly 'milder' average mental impairment for TSC1 mutations (59% versus 34%), although even the combined numbers are still too small for obtaining sufficient statistical power. The frequency of epilepsy did not seem to be very different for both groups. In addition, like in Dabora's study, there is a suggestion that 'mesodermally derived abnormalities', like renal and cardiac manifestations, were more frequent among TSC2 patients.

The differences between the groups could not be explained solely by the proportion of patients with the TSC2 contiguous gene deletion syndrome. Splitting up the data into sporadic and familial cases (taking only the index patient from families) would make a very small group of sporadic TSC1 patients (n=13). Of course it might well be possible that there is a

relation between the type of mutation, the phenotype, and the reproductive fitness of TSC patients.

Whether or not a prediction of phenotype is possible from knowledge of the mutation, a comparison of all patients probably provides the best figures for this purpose, until correction for the ascertainment bias and other biases is possible. Our data are therefore presented regardless of sporadic or familial status, and regardless of whether they were index patients or not.

Table 2.15. Comparison of number of signs and symptoms of TSC between patients with a TSC1 mutation and patients with a TSC2 mutation, sporadic and familial occurrence.

	TSC1, n=35(%)	TSC2, n=44(%)
Facial angiofibroma	21(60)	33(75)
Ungual fibroma	12(34)	10(23)
Hypomelanotic macula	33(94)	44(100)
Subependymal nodule	20(57)	29(66)
Cortical tubers	11(31)	18(41)
Epilepsy, unspecified	11(31)	20(45)
Infantile spasms	3(9)	8(18)
Other type of epilepsy	20(57)	17(39)
Mental retardation	12(34)	26(59)
Renal cysts	3(9)	7(16)
Renal angiomyolipoma	2(6)	17(39)
Cardiac rhabdomyoma	6(17)	12(27)

2.2.4 Conclusions

The Rotterdam Tuberous Sclerosis Registry described in this Chapter was originally designed to be of help in identifying the causative genes by providing accurate clinical information about the diagnosis in the participating TSC patients. Gene mapping and gene identification studies are impossible without accurate clinical information on affected family members with the disease under study, as well as unaffected relatives. In addition, families with distinctive, rare phenotypes can sometimes provide clues to the genetic mechanisms involved in genetic mutation, as is illustrated in the article describing the cloning of the TSC2 gene (The European Chromosome 16 Tuberous Sclerosis Consortium 1993).

After the cloning of both TSC genes was achieved and mutation analysis became feasible, the clinical database served studies of possible genotype-phenotype correlations, so far without clear results. It remains of interest to see whether distinctive phenotypes will emerge from larger studies, other than the contiguous gene deletion PKD-TSC phenotype. Special attention

should be paid to the stratification for true sporadic and familial cases, the latter subdivided in index patients through whom the family was ascertained and affected relatives, as well as cases due to parental mosaicism. With respect to age at onset of symptoms and signs and course of the disease, our study results confirm those of previous reports. We also identified a number of different sources of potential biases that may have played a role in all of these studies. In order to extend these studies, more specialised registries would be required, preferably in specialised follow-up clinics using extensive standardised input of data for a large number of years with a defined population of TSC patients. One problem, even in such a study population will inevitably be bias towards the more severe spectrum of TSC patients, especially of the sporadic cases. Some patient societies have used questionnaires about the problems people encounter in the course of their life with (a relative with) TSC. However also this source of information has severe drawbacks, due to incompleteness or lack of accurate medical documentation, recall bias and selective membership of the patient society and selective participation in such studies.

Acknowledgements

Rob Verhage, database manager for the department of clinical genetics, was largely responsible for the creation of the database in SQL and helped by making the queries for the analyses. Substantial work, especially in the initial stages of the project of planning and gathering the patient data, and making contact with the families, was done by my colleagues J.A. Maat-Kievit and E.C. Merckens, working on the TSC project before 1991.

2.3
Malignant pancreatic tumour within the spectrum of tuberous sclerosis complex in childhood

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Abstract

A 12-year-old boy with tuberous sclerosis complex (TSC) presented with a large retroperitoneal tumour. Exploratory surgery revealed an infiltrative tumour originating from the pancreas, with local metastases to the lymph nodes. The histologal diagnosis was a malignant islet cell tumour. Retrospectively measured pancreatic hormone levels, however, were normal. A connection between the malignancy and TSC was demonstrated by loss of heterozygosity of the TSC2 gene in the tumour. The primary mutation Q478X in this patient was identified in exon 13 of the TSC2 gene on chromosome 16.

Key words

Tuberous sclerosis complex. TSC2 gene. Loss of heterozygosity. Pancreas Islet cell tumour

Abbreviations

LOH loss of heterozygosity, MEN-I multiple endocrine neoplasia syndrome type 1, TSC tuberous sclerosis complex

Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant neurocutaneous disorder, with a birth prevalence of 1:6,000-10,000 (Osborne et al.,1991), characterized by the growth of hamartomas that can appear in virtually any organ or tissue. Diagnostic criteria most widely used are those of Gomez (1991). The most frequently affected organs are skin, brain, kidneys, eyes, and heart. About half of the patients with TSC present as new cases in their family (new mutations). Small children often present with epilepsy and mental retardation. Abdominal involvement in TSC usually involves the kidneys with bilateral multiple renal cysts, often in combination with angiomyolipomas. The frequency of renal involvement in TSC has been estimated between 40% and 80% (Gomez, 1991); in a small number of patients the liver and/or pancreas show changes like those in the kidneys. Malignant degeneration of these tumours and cysts is rare. Mutation in one of two separate genes can cause TSC. Both these genes have been isolated: TSC1 on chromosome 9q34.3 (Van Slegtenhorst et al., 1997) and TSC2 on chromosome 16p13.3 (The European Chromosome 16 Tuberous Sclerosis Consortium 1993). The clinical picture of patients with TSC1 mutations or with TSC2 mutations is very similar, perhaps indistinguishable. In principle, each family has a separate mutation, but recurrence of a particular mutation in unrelated sibships has been reported Van Slegtenhorst et al., 1997, Verhoef et al., 1998).

In this report we demonstrate the case of a young boy with TSC, presenting with abdominal pain, caused by a malignant pancreatic islet cell tumour. Mutation analysis in blood cells of the patient resulted in the identification of the germline mutation in the TSC2 gene. The involvement of the TSC2 gene in the etiology of the pancreatic tumour was shown by the demonstration of allelic loss of the non mutated allele of the TSC2 gene in tumour tissue.

Materials and methods

The index patient is described in the case report (next section).

The surgical specimen was fixed for 24 hours in 4% phosphate buffered formalin, 4μm paraffin embedded sections were stained with haematoxillin and eosin. The used antibodies were directed against chromogranin-A (polyclonal, DAKO), insulin (polyclonal, DAKO), synaptophysin (monoclonal, Boehringer), gastrine (polyclonal, DAKO), islet amyloid polypeptide (gift from van Hulst (Van Hulst et al., 1994)), somatostatin (polyclonal, DAKO), glucagon (polyclonal, DAKO) and MIB-1 (monoclonal, Immunotech). Subsequently streptavidin-biotin-peroxidase staining was done. Immunohistochemical procedures were performed as described previously (Verbeek et al., 1996).

Primer sequences for direct sequence analysis of the 41 exons of the TSC2 gene are available on request. PCR conditions for exon 13 for 100 μl volume were 10 mM Tris pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 100 μM mix of each deoxynucleotide and 2 U of Taq polymerase (Gibco BRL). For the amplification reaction of exon 13 thermal cycling conditions were 5 minutes at

94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 90 seconds at 72°C with a final elongation of 5 minutes at 72°C.
Cycle sequence reactions were performed using the ABI prism dye primer cycle sequence ready reaction kit (Perkin Elmer), gels were run on an ABI 377 automated DNA sequencer.
LOH studies were initiated by comparing DNA from peripheral leukocytes and DNA from tumour tissue, using markers D9S149 and D9S150 for the chromosome 9q34 TSC1 region (Van Slegtenhorst et al., 1997), and KG8 (intragenic in the PKD1 gene, adjacent to the TSC2 gene) and 16AC2.5 (D16S291) for the chromosome 16p13 TSC2 region (The European Chromosome 16 Tuberous Sclerosis Consortium 1993). The LOH results were confirmed by allele specific oligonucleotide hybridisation analysis (sequences available on request). Hybridisation was performed at 37°C for 1 hour, filters were washed to 0.3xSSC for 10 minutes at 37°C.

Case report

The index patient was born in 1985 as the second child from healthy, non consanguineous, parents. Pregnancy and delivery had been normal. At two years a diagnosis of TSC was made on the basis of the presence of epilepsy, hypopigmented macules, mental retardation and CT-scan abnormalities characteristic of TSC: subependymal calcified nodules, hypodense areas and left frontal atrophy. Ophthalmological examination and renal ultrasound were normal. By the age of seven years, he had facial angiofibromas, a fibrous forehead plaque and ungual fibromas of the feet. At nine years of age a large angiomyolipoma of the right kidney was embolized (both kidneys showed multiple smaller angiomyolipomas), and he had to be operated on a subependymal giant cell astrocytoma, with placement of a ventriculoperitoneal shunt.
Some months afterwards he had abdominal pains. Abdominal ultrasound showed no visible changes in his pre-existing renal TSC lesions. He had normal glucose levels, erythrocyte sedimentation rate, and liver and renal function parameters. The possibility of a psychosomatic component to his complaints was considered and a period of observation was allowed. Subsequently, an abdominal CT-scan was made, showing a highly vascularized retroperitoneal tumour, growing from either the left kidney, stomach or pancreas. Needle biopsy was considered to be too riskful. Explorative surgery proved the tumour to be of pancreatic origin, with infiltrative growth into colon and stomach. Macroscopical subtotal resection was done, including the directly neighbouring lymph nodes. Post-operative recovery was without complications. Both his parents had been completely screened for TSC in the past and had shown no signs of the disease.

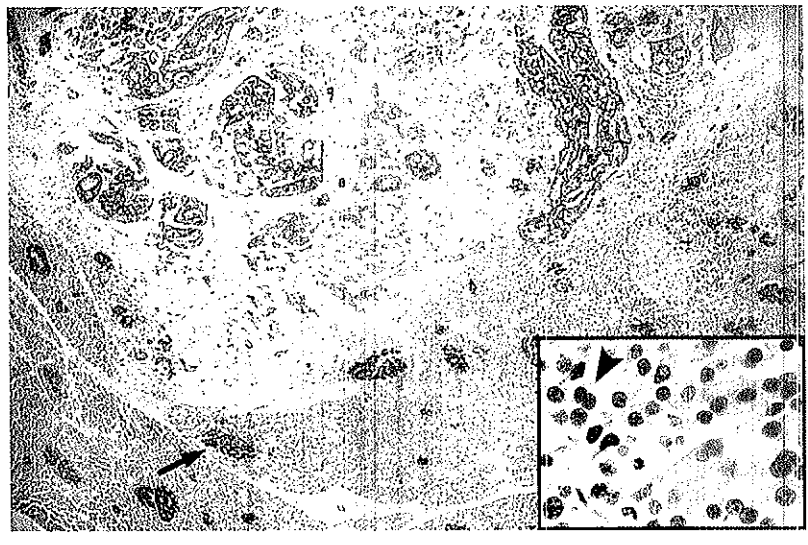
Results

Pathology showed an islet cell tumour of the tail of the pancreas (maximum length 9.5 cm), with invasive growth into the posterior wall of the stomach and transverse colon (Figure 2.6). Three of the resected lymph nodes showed metastases. Immunohistochemical staining was

positive for the neuroendocrine markers synaptophysin and gastrine, and ß-cell marker islet amyloid polypeptide, negative for insulin and somatostatin. Parts of the tumour were positive for chromogranin-A (a probable precursor for regulatory proteins), glucagon and monoclonal antibody MIB-1 (a marker for cell proliferation). Retrospectively, glucagon and glucose levels were determined in pre-operatively stored frozen plasma and found normal. The tumour thus appears to have been non-hormone producing. Complete clinical screening of the patient for other endocrine neoplasias was negative, making a diagnosis of MEN-1 syndrome improbable.

DNA-analysis for loss of heterozygosity in the tumour of either TSC gene, using chromosome 9 markers and chromosome 16 markers, showed LOH of chromosome 16 with both TSC2 markers, not with TSC1 markers (Fig 2.7). The primary, germ-line mutation in the TSC2 gene appeared to be a nucleotide substitution C into T at position 1450 in exon 13 (Q478X). The LOH result was confirmed by allele specific oligonucleotide hybridization analysis for the mutation, showing that it was the normal, wild type allele that was lost in the pancreatic tumour (Fig 2.8). The mutation was absent in DNA isolated from peripheral leukocytes of the parents and is therefore a de novo mutation.

Figure 2.6



Low magnification (42X, chromogranin-A) to demonstrate the infiltrative growth of the islet cell tumour of the pancreas in a 12 year old tuberous sclerosis patient. Note a similar staining with chromogranin-A of normal pancreatic islet cells (arrow) and tumour cells. Inset: High magnification (500X, H en E) of the tumour cells. Note the mild degree of nuclear pleomorphism and the occurrence of binucleate tumour cells (arrowhead).

Fig 2.7

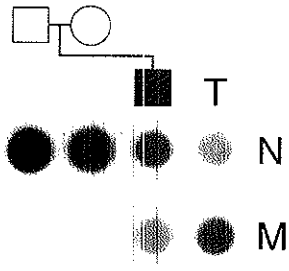


Fig 2.8

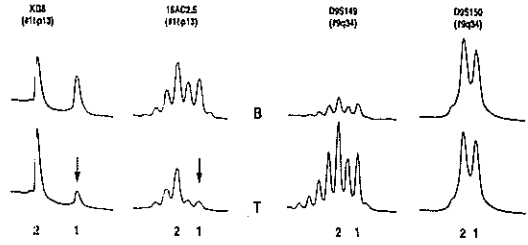


Figure 2.7: Loss of heterozygosity studies of blood (B) versus pancreas tumour (T) DNA of the index patient with TSC. For chromosome 16 (left) markers KG8 and 16AC2.5 clearly show partial loss of one allele (arrows) in DNA isolated from tumour (T) versus blood cells (B). For chromosome 9, markers D9S149 and D9S150 show no loss. (Although the overall signal in blood for D9S149 is reduced, the relative ratio of alleles 1 and 2 remains unchanged).

Figure 2.8: Demonstration of the Q478X mutation in exon 13 of the TSC2 gene in the patients blood (square) and tumour tissue (T) by allele specific oligonucleotide hybridization. N= the normal allele, which is also present in the parents (left two dots). M = mutated allele. The relative intensities of the normal versus the mutated signal are clearly reversed in the tumour tissue, indicating loss of heterozygosity of the wild type TSC2 allele.

Discussion

We present a mentally retarded boy with tuberous sclerosis who developed an unexpected malignant pancreatic tumour, with infiltrative growth and local metastases to the lymph nodes. Since radical excision was not possible, follow-up by regular CT-scan will be done, as recurrence is possible. The tumour was classified as a non-hormone producing islet cell tumour, the boy did not show clinical signs of a hormonal imbalance. Neuro-endocrine tumours in children are found in MEN-1 syndrome, which has been mapped to chromosome 11. Screening for other neuroendocrine tumours showed no other manifestations of a possible coexisting MEN-1 syndrome. In MEN tumours loss of chromosome 16p13 has not been described. To our knowledge, no other reports on malignant pancreatic tumours in children with TSC have been published. If affected, the pancreas usually shows cysts and angiomyolipomas. Islet cell tumours and gastrinoma have been incidentally reported in adult TSC patients (Davoren and Epstein 1992, Kim et al., 1995, Schwarzkopf and Pfisterer 1993). Malignancies do sometimes arise in association with tuberous sclerosis (Gomez 1988). Loss of heterozygosity has been reported in non-metastasizing hamartomas of TSC patients (Green et al., 1994). At present it is unknown how the relatively rare transition from benign hamartoma to metastasizing malignant tumour should be viewed. Little is known about the interactions between the TSC1 and TSC2 gene products, called hamartin and tuberin respectively (Van Slegtenhorst et al., 1998). The demonstration of loss of the wild type TSC-2 allele in a neuro-endocrine pancreatic tumour of this TSC patient strongly suggests a role for

TSC2 as a tumour suppressor gene in its etiology, in line with the proposed tumour suppression function of tuberin (Green et al., 1994). This loss of the non-mutated allele, or 'second hit' is considered to be a decisive step in tumorigenesis (Knudson 1971). Mutation analysis resulted in the identification of the Q478X germline mutation or 'first hit' in the patient, likely to produce a severely truncated tuberin protein, instable and/or inactive, from that allele. A naturally occurring rat strain with a germline insertion in the TSC2 gene is the 'Eker rat', which is prone to develop renal carcinoma. In vitro, tumour growth can be inhibited by introducing an active TSC2 gene into cultured tumour cells of this rat (Orimoto et al., 1996), supporting the hypothesis that the TSC2 gene is a tumour suppressor gene.
Our findings show that the TSC2 gene can be involved in tumours that are not specifically associated with TSC, and might play a more general role in the suppression of tumour growth in different kinds of tissues.

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2.4
The evolution of renal angiomyolipomas in patients with tuberous sclerosis

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Abstract

In 1986, 23 patients with renal angiomyolipomas as part of tuberous sclerosis were assessed by ultrasonography. In 1991, 20 patients in this group were re-examined with special attention paid to the renal pathological condition. Ultrasonography was performed by the same radiologist who performed the examination in 1986. Of 20 patients 7 had severe haemorrhage necessitating hospital admission (5 had a renal lesion larger than 3.5 cm. in diameter). In 2 patients the exact diameter of the renal angiomyolipomas could not be determined and they underwent nephrectomy. Three patients underwent successful selective embolisation of the bleeding angiomyolipoma. One patient died. The haemorrhage resolved spontaneously in 1 patient and treatment was not feasible. In 4 patients the lesions increased in size between 1986 and 1991.

Based on these results there is a relationship between the size of the angiomyolipomas and the risk of bleeding. Renal angiomyolipomas larger than 3.5 cm. in diameter have a substantial risk for severe haemorrhage. Some angiomyolipomas show progression. Periodic followup is mandatory every 6 months. For angiomyolipomas larger than 3.5 cm. in diameter an aggressive approach is advised. Selective embolisation is the initial method of choice.

Key words

kidney diseases, lipoma, embolisation, therapeutic

Introduction

Renal angiomyolipomas are infrequent congenital mixed tumour-like formations consisting of blood vessels, smooth muscle cells and adipose tissue. These benign lesions generally are classified as hamartomas.(Perou and Gray 1960) In a series of 8,501 necropsies, Hadju and Foote reported on 27 renal hamartomas (0.3%).(Hadju and Foote 1969) Another series reported a frequency of 3% among all solid renal masses.(Mazeman et al. 1980) Renal angiomyolipomas occur in 2 forms: isolated or as part of the tuberous sclerosis complex. The isolated form is usually small and asymptomatic, and occurs predominantly in women between decades 4 and 7 of life. The combined form is encountered in 40 to 80% of the patients with tuberous sclerosis. Under these circumstances the tumours are usually described as small, multiple and bilateral, and cause no symptoms (van Baal 1987). However, both forms may become large and symptomatic.

Pain is the most frequent clinical feature and, as most other symptoms, is caused by haemorrhage in the tumour. The fact that these larger tumours are prone to bleeding is not surprising, since they are a highly vascular agglomeration of abnormal blood vessels. The high degree of vascularization, structured rigidity, inelasticity and tortuosity of blood vessels are predisposing factors that lead to spontaneous bleeding in the tumour. In 10% of the cases the bleeding may be so severe that it leads to hypovolemic shock(Tuberous sclerosis 1979).

In general, hemorrhagic complications of these renal angiomyolipomas are not noted before the second decade of life, while the risk of bleeding after this period is considered low. Moreover, renal angiomyolipomas are classified as hamartomas and their growth potential in adults is considered low. Nevertheless, haemorrhage does have serious implications, especially when, in a patient with tuberous sclerosis, the renal function is already compromised by multiple bilateral angiomyolipomas and cysts (van Baal 1987).

Apart from some case reports, there is no good documentation of the evolution of the combined renal angiomyolipomas during a longer period in adults. However, for the implications of treatment it is mandatory to have an indication of the risk of haemorrhage and the growth potential of these hamartomas. We present a 5 year follow-up study of 20 patients with tuberous sclerosis and renal angiomyolipoma.

Materials and methods

In 1986 we performed a study of 30 patients with tuberous sclerosis and their 60 parents. Tuberous sclerosis was diagnosed according to the criteria described by Gomez.(Tuberous sclerosis 1979) Of the patients 23 had renal angiomyolipomas and 9 had multiple renal cysts, including 6 in combination with angiomyolipomas. The renal disease was diagnosed by ultrasonography performed by 1 radiologist (N.S.). The examinations performed in 1986 included patient history (with special attention to neurological, pulmonary and renal pathological conditions), family history, clinical examination (skin and eyes, including funduscopy) and radiological examination (computerized tomography [CT] of the brain, x-ray

of the thorax and spine, sonography of the liver and kidneys, and cardiac ultrasound) (van Baal 1987).

In 1991 a follow-up study of these patients was initiated. A history was obtained with special attention to renal symptoms. Ultrasonography of both kidneys was performed by the same radiologist who performed the test in 1986. Of the 23 patients with previously recorded angiomyolipomas 19 entered this study. One patient died of renal bleeding combined with the severe neurological involvement with tuberous sclerosis. Data regarding the renal disease were sufficient to include these patients in this study. The remaining 4 patients refused to cooperate, mainly because the mental disability made the trip to our hospital an insurmountable burden. In 1991 all except patient 20 were treated and followed for the tuberous sclerosis elsewhere. For this reason, additional laboratory examinations were not performed.

Results

The personal data of all patients are summarised in Table 2.16. The male-to-female ratio was 3:7. Patient age varied from 13 to 54 years (mean age 27.6 years). Seven patients (35%) had serious renal haemorrhage necessitating hospital admission (tables 2.16 and 2.17). Complications of treatment included transient pain, nausea, fever and increased serum creatinine levels in patient 1; persistent fever and abscess of the left kidney successfully drained percutaneously in patient 2 and hypertension (well controlled by medication, caused by renal insufficiency) and renal osteodystrophy (managed by dietary measures) in patient 20.

Table 2.16. Patient data, symptoms in 1991, results of radiological examination, and haemorrhagic complications

PtNo-sex-age(1991)	Ultrasonography 1986	Ultrasonography 1991	Change in symptoms	Haemorrhage
1-F-27	Multiple bilat. angio-myolipomas. rt. (3.8)	Same	-	Yes
2-F-40	Rt. (4.2 and 5) and lt. (4) cysts	Same	-	Yes
3-F-39	Rt. (3.0 and 1.5) cysts. post lt. nephrectomy	Same	-	Yes
4-F-24	Multiple bilat. angiomyolipomas lt. (1.5) cysts	Multiple bilat. angiomyolipomas. lt. (2.2) cysts	-	No
5-M-21	Multiple bilat. small angiomyolipomas cysts	Multiple bilat. small angiomyolipomas. rt. (2.5) and lt. (3.5) cysts	-	Yes
6-M-22	Multiple bilat. angiomyolipomas, rt. (1.2) cysts	Multiple bilat. angiomyolipomas rt. (2.5) cysts	-	No

7-M-45	Rt. (2.2 and 3.3) and lt. (2.9) cysts	Same	Dull flank pain	No
8-F-16	Multiple bilat. angiomyolipomas cysts, rt. large cyst (4.5)	Multiple bilat. small angiomyolipomas, cysts, large cyst disappeared.	-	Yes
9-M-13	Multiple bilat. small angiomyolipomas, cysts	Same	-	No
10-F-16	Multiple bilat. small angiomyolipomas, cysts	Same	-	No
11-F-13	Multiple bilat. small angiomyolipomas	Same	-	No
12-F-42	Bilat. small angiomyolipomas	Same	-	No
13-F-17	Multiple bilat. angiomyolipomas rt. (5.5) and lt. (4.2) cysts	Died of large retro-peritoneal haematoma and macroscopic haematuria, Side unknown in 1990	Died	Yes
14-M-20	Multiple bilat. small angiomyolipomas	Same	-	No
15-M-25	Multiple bilat. small angiomyolipomas	Same	-	No
16-F-54	Bilat. small angiomyolipomas	Same	-	No
17-F-30	Multiple bilat. small angiomyolipomas	Same	Anaemia	No
18-F-16	Multiple bilat. small angiomyolipomas lt. (1.7) cyst	Multiple bilat. small angiomyolipomas, lt. (4.9)	Macroscopic haematuria	No
19-F-29	Multiple bilat. small angiomyolipomas	Same	-	No
20-F-42	Post lt. nephrectomy rt. (4 and 3) cysts	Same	Renal function disturbance	Yes

Parentheses indicate largest size in centimetres.

At the time of haemorrhage patients 1, 2, 5. 8 and 13 had renal lesions larger than 3.5 cm. in diameter. Patient 13 died of complications of the bleeding in 1990. Patients 3 and 20 underwent nephrectomy for large bleeding angiomyolipomas before 1986. The exact diameter of the bleeding lesions could not be determined. In patient 20, 2 renal angiomyolipomas in the remaining kidney were treated with selective embolisation in 1987 because of growth tendency. After this treatment the patient had no signs of haemorrhage during follow-up. However, the pre-existing renal function disturbances were aggravated. Hypertension and signs of renal osteodystrophy developed, which were managed by medication and diet.

Table 2.17. Clinical data of patients with hemorrhaghic complications.

Pt.No-Yr.	Symptoms	Therapy
1 - 1988	Colicky rt. flank pain, anaemia	Selective embolisation of bleeding angiomyolipomas rt. Side
2 - 1988	Severe rt. flank pain, anaemia	Selective embolisation of bleeding angiomyolipomas rt. Side
1989	Colicky lt. Abdominal pain	Selective embolisation of bleeding angiomyolipomas lt. side
3 - 1981	Pain, anaemia, hypovolemic shock	Lt. Nephrectomy
5 - 1990	Pain, anaemia, hypovolemic shock	Selective embolisation of bleeding angiomyolipomas lt. side
8 - 1990	Abdominal tenderness, anaemia	Exploratory laparotomy with biopsy of bleeding renal lesion
13 - 1990	Hypovolemic shock, signs of renal haemorrhage	Died
20 - 1978	Pain lt. side, hypovolemic shock	Lt. nephrectomy (in 1986 selective embolisation of 2 large angiomyolipomas on rt. side)

Patients 1, 2 and 5 underwent successful selective embolisation of bleeding angiomyolipomas in 1987 and 1988. Patient 2 underwent a second successful embolisation of a bleeding angiomyolipoma on the contralateral side in 1988. During the short follow-up since embolisation none of the 3 patients had recurrent haemorrhage. Apart from a temporary increase in serum creatinine levels, the renal function remained undisturbed and the patients had no symptoms. Patient 8 was hospitalised with the diagnosis of acute appendicitis and underwent surgery. Retroperitoneal haemorrhage was found to be the cause of the complaints. Specimens from a renal tumour confirmed the diagnosis of angiomyolipoma. The haemorrhage resolved spontaneously. Selective renal arteriography 2 months later showed multiple small angiomyolipomas, not amenable to selective embolisation. A total of 13 patients had no serious haemorrhage. In 12 patients the renal angiomyolipomas were smaller than 3.5cm. During follow-up patient 18 had a large angiomyolipoma causing periodic macroscopic haematuria. At examination in 1991, 4 patients had symptoms of kidney and/or renal function disturbances, including patient 20, who had symptoms of renal insufficiency after selective embolisation.

All ultrasonographic studies performed in 1991 were compared with the echographic results in 1986 and related with the symptoms. In 14 patients (70%) there was no apparent change in the size of the lesion. Of these patients 4 (29%) had severe haemorrhage (patients 1, 2, 3 and 13), and patient 13 died of a large retroperitoneal haematoma and macroscopic haematuria, side unknown, in 1990.

In 1986 a large right renal cyst apart from multiple bilateral small cysts and angiomyolipomas was recorded in patient 8. Because this cyst had disappeared on ultrasonography in 1991, it is highly probable that the bleeding had occurred in the large cyst. On ultrasonography patients

4, 5, 6 and 18 (20%) had angiomyolipomas that increased in size. On comparison of the ultrasonographic studies in patient 5 in 1986 and 1991, the radiologist admitted the possibility of having missed 1 of the larger angiomyolipomas in 1986, which could have been attributed to the fact that this hyperechoic lesion was located predominantly outside the normal contour of the kidney within the perirenal fat tissue of hyperechoic consistency, in which case the actual size of the angiomyolipoma may be difficult to measure with the less sensitive device used in 1986.

Discussion

The number of patients in this prospective study is too small to warrant any statistical significance. However, this study strongly suggests that renal angioinyolipomas larger than 3.5 cm. in diameter have a substantial risk of severe haemorrhage. This conclusion is in accordance with the retrospective study of Oesterling et al. (1986).

Renal angiomyolipomas are considered as hamartomas. Hamartomas in adults are usually considered not to have growth potential. Our data show an increase in size of the angiomyolipomas in 4 patients (20%). The number of patients is small and the follow-up is possibly too short to predict the growth potential of the unchanged angiomyolipomas during a longer period. Nevertheless, because the risk of bleeding of the larger angiomyolipomas is considerable, we suggest periodic sonographic follow-up (every 6 months) of all patients with tuberous sclerosis and proved renal angiomyolipomas. All patients had renal haemorrhage after the age of 10 years. Most patients had complications of haemorrhage in the second decade of life. One patient died of complications of tuberous sclerosis. The patient was severely mentally retarded, and had multiple gastric and renal haemorrhages. Operative treatment was not feasible. Most angiomyolipomas in patients with tuberous sclerosis are multiple and bilateral. The combination with multiple renal cysts (9 patients in our series had renal cysts combined with renal angiomyolipomas, Table 2.16) may cause compression of functional kidney tissue. Therefore, it is important in case of hemorrhagic complications to safeguard as much functional renal tissue as possible.

Two patients in this series lost the kidneys because of a benign tumour. Both patients underwent emergency surgery with the incorrect diagnosis of a malignant tumour. With the introduction of CT and magnetic resonance imaging the diagnosis of angiomyolipoma can always be made mainly due to the fat component of renal angiomyolipomas (Bosniak 1981). Renal ultrasonography has less sensitivity and less specificity in the diagnosis of renal angiomyolipomas than the more advanced methods, such as magnetic resonance imaging and CT, and may underestimate the actual size of the angiomyolipomas. When the fatty lesion projects outside the contour of the kidney, discrimination from the perirenal fatty tissue may be difficult. Some patients have so many angiomyolipomas that ultrasonography indicates a confluence of angiomyolipomatous tissue throughout the kidney, in which case the size of a separate angiomyolipoma is difficult to estimate. When in doubt, the aforementioned, more invasive and expensive methods will be used. In established cases, however, ultrasonography

is a safe method for follow-up purposes. The estimated prevalence of tuberous sclerosis in western society is 1:10,000 (Wiederholt, Gomez, and Kurland 1985; Lindenbaum 1985). During the last 5 years more cases of so-called incomplete forms of tuberous sclerosis have been registered with a normal life expectancy. Therefore, the number of patients with a slight degree of tuberous sclerosis will increase. Renal involvement with tuberous sclerosis will be present in at least 50% of these patients. For this reason, acquaintance with the possible hemorrhagic complication and the differential diagnosis from malignancies are increasingly important. In case of severe haemorrhage there are several treatment modalities, such as selective transcatheter embolisation and kidney sparing extirpation of the tumour. Until now there was no consensus on whether larger angiomyolipomas needed preventive treatment, such as embolisation or limited resection. However, our results suggest an aggressive approach towards large angiomyolipomas. Therefore, angiomyolipomas larger than 3.5 cm. should be treated prophylactically. embolisation and extirpation of the angiomyolipomas have their own indications depending upon the angiographic pattern and technical feasibilities. Complications have also been described (van Baal et al. 1990; Earthman, Mazer, and Winfield 1986). We have performed selective embolisation with satisfactory results (van Baal et al. 1990). There are a few reports on selective embolisation in patients with haemorrhagic renal angiomyolipomas. Follow-up varied from 6 to 14 months. Apart from the incomplete embolisation necessitating an operation within 1 week after initial treatment, there was no recurrent haemorrhage during follow-up (Sanchez et al. 1985; Zerhouni et al. 1984; Adler, Greweldinger, and Litzky 1984). In our series 9 patients had multiple renal cysts as part of the tuberous sclerosis complex. Renal cysts mimicking polycystic kidney disease are frequently noted in patients with tuberous sclerosis and are the main cause for renal insufficiency as a complication of tuberous sclerosis (Oesterling et al. 1986). Recent reports described patients with a clear diagnosis of tuberous sclerosis of families in which apparently isolated and clinically typical autosomal dominant adult polycystic kidney disease was diagnosed in first degree relatives (Kandt et al. 1992). In a patient with only renal cystic lesions, the differential diagnosis between adult polycystic kidney disease and tuberous sclerosis cannot be made on ultrasound imaging alone. Linkage studies have provided strong evidence that tuberous sclerosis is a disease with genetic locus heterogeneity and that 1 of the major genes is located on chromosome 16, the same region in which the adult polycystic kidney disease gene was localised. Identification of the genes and/or gene mutations in adult polycystic kidney disease and tuberous sclerosis will help us to understand the morphology, biological behaviour and possible interrelationship of renal lesions in tuberous sclerosis and adult polycystic kidney disease.

Conclusions

In patients with tuberous sclerosis several conclusions may be drawn. Renal angiomyolipomas larger than 3.5 cm in diameter have a substantial risk for severe haemorrhage. These complications are encountered in decades 2 and 3 of life. Some renal

angiomyolipomas show progression. Once the diagnosis of renal angiomyolipomas has been established, follow-up by ultrasonography is mandatory and feasible every 6 months. When the angiomyolipoma becomes larger than 3.5 cm. in diameter, preventive treatment is necessary.

2.5
Familial cylindromatosis mimicking tuberous sclerosis complex and confirmation of the cylindromatosis locus, CYLD1, in a large family

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Abstract

A large Dutch family had been known for many years to be affected with skin tumours labelled as adenoma sebaceum, which were inherited in an autosomal dominant fashion. Since this skin sign is considered pathognomonic for tuberous sclerosis complex, the condition in the family was labelled accordingly, in the absence of further clinical features of tuberous sclerosis complex-like mental retardation or epilepsy. The skin changes started at early puberty with small eruptions around the nose and progressed to larger tumours, with considerable variation in severity. Some affected members had required plastic surgical reconstruction following excision.

Linkage analysis in this family was performed for the two chromosomal regions involved in tuberous sclerosis complex on chromosomes 9q34 and 16p13, but no positive linkage was found. On critical re-evaluation of the clinical and pathological data and renewed assessment, the working diagnosis was changed to autosomal dominant cylindromatosis.

The recently published candidate region for cylindromatosis on chromosome 16q12-13 was subsequently proven to be positively linked with a lod score of 3.02 with marker D16S308. Review of pathological specimens confirmed the diagnosis of cylindromatosis. DNA analysis of tumour tissue showed loss of heterozygosity for the cylindromatosis CYLD1-locus. These results confirm the candidate locus for cylindromatosis on chromosome 16q12-13.

Keywords

tuberous sclerosis; cylindromatosis; CYLD 1 gene; chromosome 16

Introduction

Hereditary multiple epithelioma, or cylindromatosis (MIM No 132700), is a rare autosomal dominant disease, characterised by the appearance of multiple skin adnexal tumours with different histological descriptions and names. The primary localisations of these usually benign lesions are the head and neck. The genetic aspects of this disease were comprehensively reviewed by van Balkom and Hennekam (1994), who used the name dermal eccrine cylindromas for the condition. Recently, the gene for familial cylindromatosis, referred to as CYLD1, was mapped to the long arm of chromosome 16, band 16q12-13, in a genome search involving two large families(Biggs et al. 1995). So far, there is no evidence for locus heterogeneity.

The skin abnormalities of cylindromatosis may be confused with some of the manifestations of tuberous sclerosis complex (TSC), previously designated epiloia or Morbus Bourneville-Pringle. TSC is an autosomal dominant disease with a prevalence of between 1 :6000 and 1: 10 000 at birth, characterised by the presence of hamartomas that can appear in - any organ system. The organs most often involved are the skin, central nervous system, eyes, kidneys, and heart. The clinical expression of TSC is extremely variable and occasionally families can have a predominance of skin manifestations. Two genes are involved in tuberous sclerosis complex. Both genes have recently been isolated, the TSC1 gene on the long arm of chromosome 9, band 9q34 (Slegtenhorst van et al. 1997) and the second gene, TSC2, on the short arm of chromosome 16, band 16p13.3 (The European Chromosome 16 Tuberous Sclerosis Consortium 1993).

We describe a family in which a clinical diagnosis of tuberous sclerosis complex was made on the basis of the presence of so-called "sebaceous adenoma", a skin sign pathognomonic for the disease, in several of its members. Linkage analysis of the two regions for the TSC1 and TSC2 genes, and subsequently the candidate region for the CYLD 1 gene, as well as review of the skin pathology and loss of heterozygosity studies, were performed. These enabled us to resolve this differential diagnostic problem and confirm the candidate region of CYLD1 on chromosome 16q12-13.

Patients and methods

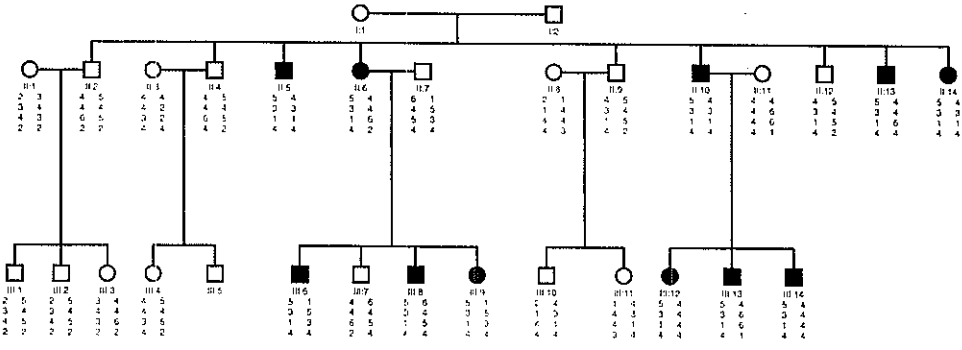
A total of 27 members of family T6065, of which 10 members are affected, were sampled (fig 2.9). The family was recruited through index patients II.6 and II.10. All participating family members were seen by a clinical geneticist (CSS) at the time of venepuncture and completed a questionnaire on their state of health, with special focus on skin signs and other possible signs of tuberous sclerosis complex.

EDTA blood samples were collected from affected and unaffected subjects and partners, initially for linkage analysis for both the TSC 1 and TSC2 regions. DNA extraction from peripheral leucocytes was performed according to standard procedures. Linkage analysis was undertaken mainly using microsatellite markers with the linkage computer program MLINK.

Chromosome 9q markers used flanking the TSC1 region were D9S149, D9S66, and D9S114. For chromosome 16p, markers 3'HVR, KG8, 16AC2.5, and SM7, flanking the TSC2 gene, were tested. KG8 is a marker in the adjacent PKD1 gene showing 0% recombination with the TSC2 gene. Subsequently, the cylindromatosis candidate region on chromosome 16q was tested with markers D16S411, D16S304, D16S308, and D16S419. Calculations were performed using a gene frequency of 0.1%, a penetrance of 80%, and a 1% phenocopy rate. Allele frequencies were presumed equal.

Some of the previously removed and analysed tumours were re-evaluated histologically and investigated for loss of heterozygosity for both TSC loci and for the familial cylindromatosis CYLD1 gene candidate region (Biggs et al. 1995, 1996). For the LOH studies, DNA isolated from blood and from tumour tissue from subject II.5 was run on an ALF automated sequencer (Pharmacia), using markers D9S66 (TSC1 locus), 16AC2.5 (D16S291, TSC2 locus), and D16S411 (CYLD1 candidate region).

Figure 2.9: Pedigree of family T6065



In the pedigree the haplotypes of the markers of the 16q12-13 region are depicted. The markers used from the CYLD candidate region from proximal to distal (top to bottom) D16S411, D16S304, D16S308 and D16S419 (affected haplotype 5-3-1-4).

Results

The multiple dermal tumours in family T6065 were mostly restricted to the face, neck, and scalp, and sometimes the ears. Clinical records and re-evaluation showed no evidence for the presence of hypomelanotic macules, shagreen patches, fibrous forehead plaques, or (peri)ungual fibromas in any of the affected family members. The age of onset of the skin signs was between 10 and 20 years of age in most subjects, with slow progression, sometimes more rapid during puberty, pregnancy, or in periods of increased psychological stress (fig 2.10A). Patient II.5 had tumours removed recently, which were sent in for pathological review (VDV). Patient II.6 (fig 2.10B) had a clinical diagnosis of facial adenoma sebaceum in 1986. CT scan of the brain and skull X-ray were normal. In 1990, again the diagnosis of

"typical Morbus Pringle-Bourneville" (TSC) was made by one of her physicians. Pathological analyses of her tumours, reviewed on more than one occasion, gave different diagnoses of basalioma, trichoepithelioma, or cylindroma. Patient II.1 0 (fig 2.10C) had a clinical diagnosis of "Morbus (Bourneville)-Pringle" (TSC) in 1977. On several occasions skin tumours had been removed and invariably histology had been reported as trichoepithelioma. He underwent clinical examination in 1986 of his brain (CT scan), kidneys (ultrasound), eyes, skin, and teeth. Apart from his skin lesions, then referred to as "adenoma sebaceum", he had no other possible signs of tuberous sclerosis complex. In this patient, some lesions were so severe that skin grafts were necessary after removal. Patient II.14 had a previous clinical diagnosis of "Morbus Pringle-Bourneville" (TSC).

Two tumours removed in 1988 were diagnosed as eccrine spiradenomas and three others removed in 1990, 1992, and 1994 as cylindromas. The tumour removed in 1990 was recently re-examined (VDV), confirming the diagnosis of cylindroma (fig 2.11). None of the affected family members had suffered from medical complications characteristic of tuberous sclerosis complex, like epilepsy, mental retardation, renal cysts, or renal angiomyolipomas.

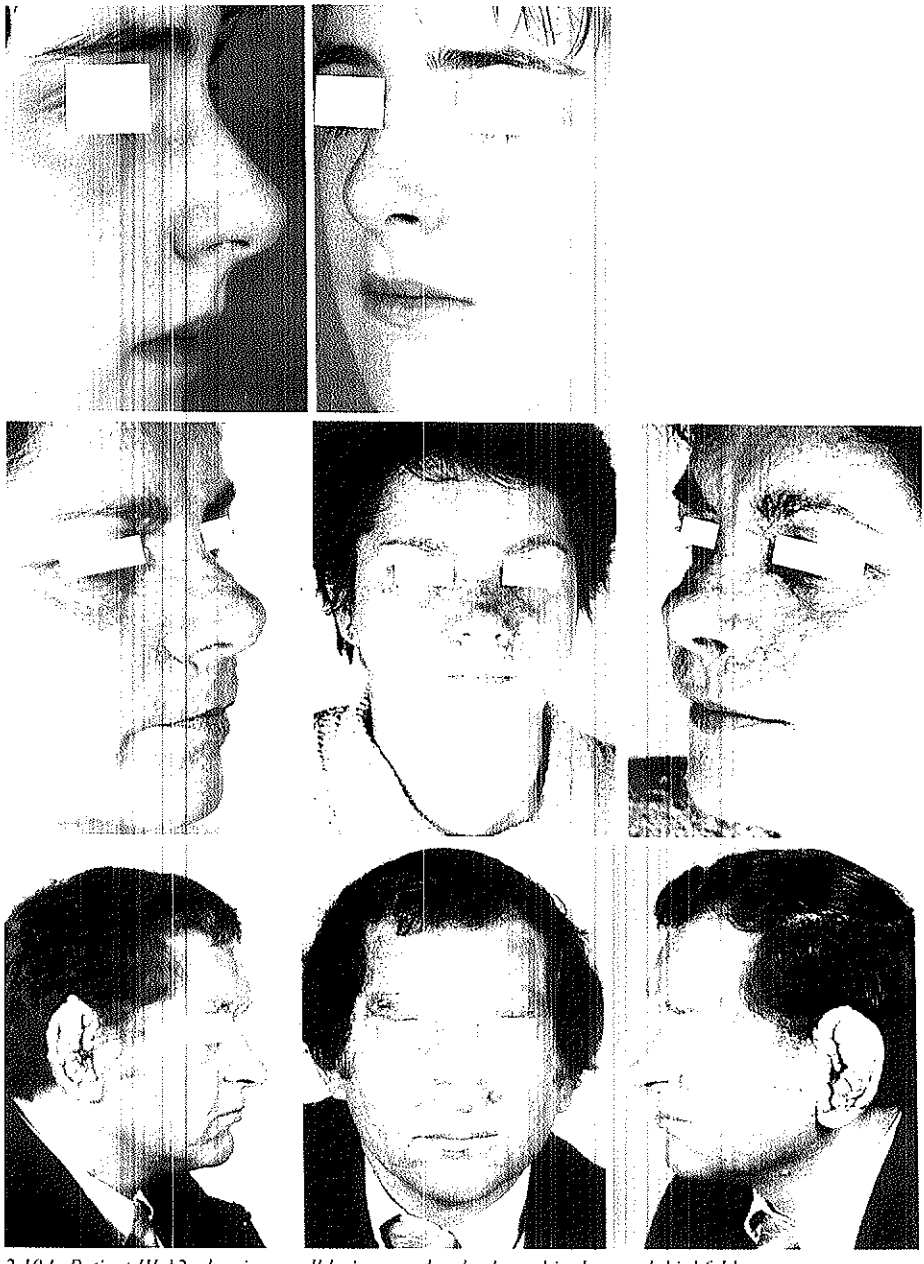
Lod scores proved negative for both the TSC1 and the TSC2 locus using flanking markers (table 2.18). Subsequent linkage for the candidate region for familial cylindromatosis on chromosome 16q12-13 gave a highest lod score of 3.02 with marker D16S308, with no recombinations in affected subjects. The affected haplotype was, proximal to distal, 5-3-1-4 (D16S411, D16S304, D16S308, D16S419).

Table 2.18: MLINK analysis for family T6065 for the regions containing the TSC1 (chromosome 9q34), TSC2 (chromosome 16p13.3), and the candidate region for the CYLD1 gene (chromosome 16q12-13).

Theta	0%	5%	10%	20%	30%	40%
Chromosome 9						
D9S149	-3.92	-2.00	-1.32	-0.66	-0.29	-0.08
D9S66	-5.09	-2.38	-1.46	-0.61	-0.24	-0.06
D9S114	-5.23	-2.53	-1.57	-0.66	-0.25	-0.06
Chromosome 16p						
3'HVR	-6.84	-2.25	-1.24	-0.39	-0.10	-0.02
KG8	-0.01	-0.01	-0.01	-0.01	-0.002	-0.001
16AC2.5	-6.77	-1.87	-1.01	-0.30	-0.07	-0.01
SM7	-2.85	-0.61	-0.13	0.18	0.17	0.06
Chromosome 16q						
D16S411	1.69	1.54	1.38	1.04	0.66	0.25
D16S304	2.21	2.00	1.78	1.30	0.77	0.24
D16S308	3.02	2.75	2.46	1.84	1.14	0.39
D16S419	2.28	2.06	1.84	1.34	0.80	0.26

Gene frequency 0.01%, penetrance 80%, phenocopy rate 1%, allele frequencies presumed equal . Markers are ordered top to bottom, proximal to distal. The maximum LOD score of 3.02 is attained at D16S308.

Figure 2.10A(top),B(middle),C(bottom)



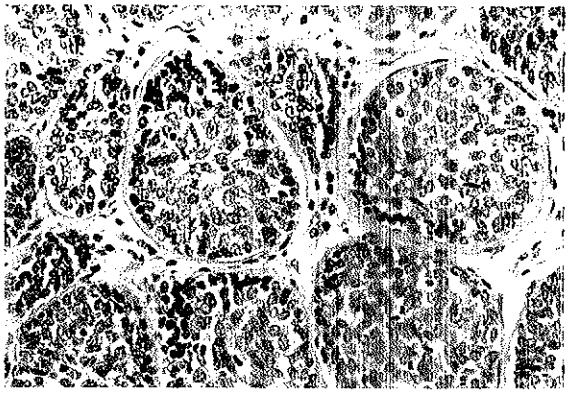
2.10A: Patient III:12, showing small lesions on the cheeks and in the nasolabial folds.

2.10B: Patient II:6, showing facial tumours in the nasolabial fold, mimicking angiofibroma. The histological diagnosis was cylindroma.

2.10C: Patient II:10, with large tumours in the nasolabial fold, on the cheeks and on both ears.

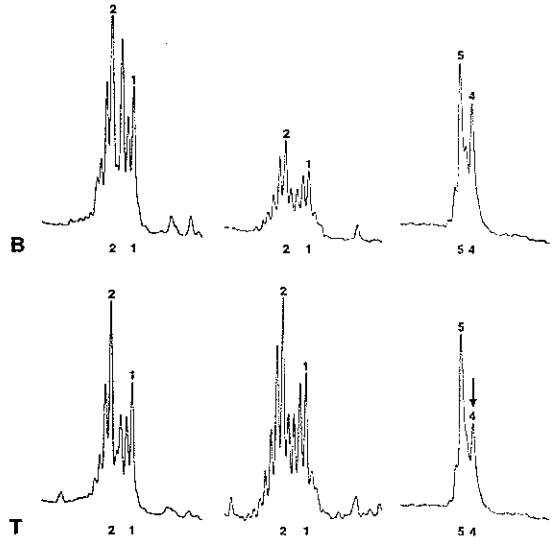
Pathological review of three tumours from the scalp of patient II.5 resulted in a histopathological diagnosis of trichoepithelioma for all three tumours. In this tumour tissue, loss of heterozygosity for the chromosome 16q marker D16S411 could be shown, with loss of the normal number 4 allele (fig 2.12). The other markers on chromosome 16q were uninformative in this subject.

Figure 2.11: Pathological analysis of one of the tumours of patient II:14.



Lobules of basophilic epithelial cells, surrounded by hyaline membranes, are present. Diagnosis: cylindroma(H&E, 280x).

Figure 2.12: Loss of heterozygosity(LOH) studies of blood (B) versus tumour (T) DNA of patient II:5.



Left and middle: chromosome 9q34(TSC1) marker D9S66 and chromosome 16p13(TSC2) marker 16AC2.5(D16S291) respectively, showing no LOH. Right: Chromosome 16q12-13(CYLD1) marker D16S411, showing partial loss of the 'normal, non affected' number 4 allele (arrow), in tumour.

Discussion

The clinical picture in this family gave rise to confusion about the exact diagnosis, despite the fact that in some of the pathological reports a diagnosis of cylindromatosis had been correctly made. The tumours had been associated with adenoma sebaceum and therefore with tuberous sclerosis complex.

Familial multiple cylindromatosis and multple epitheliomas are considered one clinical entity. The two main manifestations, cylindroma and (tricho)epithelioma, have been reported within one family and in a single person.. The most severe type of manifestation is the so called "turban tumour", as was reported by Biggs et al. (1995). In the present family, T6065, an "unusual type of tuberous sclerosis complex, manifesting itself only in the skin, as sebaceous adenoma" was apparent. The localisation of these tumours was reported to be predominantly in the nasolabial folds, as is also considered characteristic of tuberous sclerosis complex. Some more severely affected subjects had tumours elsewhere (fig 2.10). Family members had not been further analysed clinically because the condition was known to be familial and considered to be mild, apart from the severe cosmetic problems it was causing. Patients II.6 and II.10 had been investigated fully for other signs of tuberous sclerosis, but no other manifestations of tuberous sclerosis complex had been found. Removal or biopsy, followed by histological analysis, had been performed by a plastic surgeon or dermatologist in several members of the family. On these occasions, the pathological findings had been interpreted as "in agreement with a diagnosis of tuberous sclerosis". The term sebaceous adenoma, suggesting a skin adnexal origin, is now considered a misnomer for angiofibroma as found in definite cases of tuberous sclerosis complex. The tumour is not derived from the eccrine tissue of the sebaceous gland, but it contains cells with fibroblastic, vascular, and neuronal properties (Nickel and Reed 1962; Ishibashi et al. 1991) more in line with the hamartomatous changes that are seen else- where in the body in tuberous sclerosis complex. The term sebaceous adenoma could have contributed to the diagnostic confusion in this family. The name facial angiofibroma is proposed as a more descriptive and distinguishing term.

After the exclusion of linkage to either the TSC 1 or TSC2 locus, analysis using polymorphic markers of the chromosome 16q12-13 region gave convincing evidence that the diagnosis in this family should be familial cylindromatosis. Our results independently confirm the localisation of the proposed CYLD 1 locus on chromosome 16q12-13, with a maximum lod score of just over 3.0 with marker D16S308. The maximum lod score in the original article was attained with marker D16S304 (Biggs et al. 1995).

Not until the linkage analysis had excluded both candidate regions for tuberous sclerosis complex did a renewed look at the family data permit a change in the suspected diagnosis. In addition to the linkage analysis results, the observation of loss of heterozygosity of the relevant chromosome 16q region in lesional tissue confirmed that the CYLD 1 candidate region on chromosome 16q contained the locus for cylindromatosis in this family. This case may serve to illustrate the need for careful verification of the diagnosis in the case of genetic counselling for diseases that are known for their clinical variability.

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CHAPTER 3

GENOTYPES OF TUBEROUS SCLEROSIS COMPLEX

3.1
Review of the literature

Since the identification of the TSC1 and TSC2 genes (van Slegtenhorst et al. 1997; The European Chromosome 16 Tuberous Sclerosis Consortium 1993), many reports describing mutations TSC patients have been published, providing insight into the spectrum of mutations. In order to facilitate mutation detection and promote communication between DNA research and diagnostic laboratories, an international TSC mutation database (http://exped.bwh.harvard.edu/tsc/) was set up. By February 2001, a total of 360 different mutations had been reported to the database: 107 (30%) in the TSC1 gene, 253 (70%) in the TSC2 gene. The mutations are dispersed over the TSC1 and TSC2 genes known types of mutations are identified: missense (only 2 in TSC1, versus 47 in TSC2), non-sense, frame-shift and splice-site mutations. To date, no true "mutational hot spots" (clustering of mutations in a specific domain or region in a gene) have been found, although a few recurrent mutations have been reported. Some differences between the TSC1 and the TSC2 gene have been found with respect to the type of mutations. In contrast to the TSC1 gene, large insertions and deletions have been detected in the TSC2 gene. Some of those deletions extend into the PKD1 gene, which is involved in the adult type autosomal dominant polycystic kidney disease (ADPKD), leading to the contiguous gene deletion syndrome of TSC/ADPKD. The results of comprehensive screening of both genes with current mutation screening methods like DGGE (Dabora et al. 1998) or HPLC (Benit et al. 2000; Choy et al. 1999) with large deletion screening by e.g. long-range PCR or FISH analysis for the TSC2 gene, yield a causative mutation in 70%-83% of patients (Jones et al. 1999; Dabora et al. 2001). is the difference in ratio of TSC1 versus TSC2 mutations between familial cases of TSC and sporadic patients. In familial cases, several studies show a roughly equal distribution between TSC1 and TSC2 related families, while the majority of the mutations in sporadic patients are identified in the TSC2 gene (about 80%). A similar situation is found in other diseases, like e.g. hereditary breast/ovarian cancer, in which a majority of mutations is found in the BRCA1 gene compared to BRCA2 (see the BIC-database: http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic). A possible contributing factor to this difference in distribution is, that an averagely more severe phenotype of TSC2 related disease could result in decreased reproductive fitness, and therefore less families. The two TSC genes are non-homologous, but it has been shown that their proteins tuberin and hamartin interact as part of a protein complex (Plank et al. 1998; van Slegtenhorst, et al. 1998). Attempts have been made to address the question of a possible genotype/phenotype correlation. The literature about distinguishable phenotypes has so far been conflicting, apart from the TSC/ADPKD phenotype caused by distortions in the chromosome 16 TSC2/PKD1 region (Brook-Carter et al. 1994). In some articles a comparatively milder phenotype, especially with regard to mental retardation, is claimed for TSC1 related disease (Jones et al. 1999; Jones et al. 1997; Dabora et al. 2001). In our own study population, there was no

evidence for a genotype/phenotype correlation as the differences were not significant (van Slegtenhorst et al. 1999).

In this chapter we present several papers describing mutations in TSC patients and families. In our laboratory, mutation analysis of the TSC genes is performed by several techniques. Fluorescent in situ hybridisation (FISH), with a combination of TSC2 specific cDNA and cosmid clones as molecular probes, is used to detect large abnormalities of the chromosome 16p TSC2 region, resulting in the identification of the larger (megabases sized) deleterious mutations leading to the TSC/ADPKD contiguous gene phenotype. Subsequently, Southern blotting analysis, using TSC2 specific cDNA clones as probes, screens for smaller (kilobases sized) abnormalities. Point mutations are identified by polymerase chain reaction (PCR) based techniques like single strand conformation polymorphism (SSCP) or denaturing gradient gel electrophoresis (DGGE), followed by DNA-sequencing of aberrant patterns for confirmation. The advantage of PCR based techniques is that rather a small amout of DNA is required to screen all coding (over 60) exons of the TSC1 gene and the TSC2 gene. In our laboratory, an allele specific oligonucleotide (ASO) hybridisation assay is used for family studies. The ASO assay is a robust and reliable technique, with the additional advantage that low levels (10-20%) of somatic mosaicism in mildly affected or asymptomatic individuals in families can be detected (Chapter 3.7).

Chapter 3.2 describes comprehensive mutation analysis of the TSC1 gene. Interesting case reports, published at the beginning of the TSC2 mutation analysis era, are presented in Chapter 3.3, decribing one of the first point mutations, Chapter 3.4, in which a colon carcinoma in a TSC patient could be shown to be unrelated to TSC, and Chapter 3.5, describing the first reported recurrent mutation in the GAP-related domain of TSC2. The issue of somatic as well as gonadal mosaicism is highlighted in Chapters 3.6 and 3.7. Mosaicism is a phenomenon well known in cytogenetics and has been well reviewed by Hall (1988). For TSC, the occurrence of mosaicism was proposed in reaction to the publication of two TSC families seemingly showing non-penetrance (Connor, Stephenson, and Hadley 1986; Hall and Byers 1987). The first proof of mosaicism in TSC by molecular analysis was provided by our group (Verhoef et al. 1995). Other investigators showed somatic mosaicism in 7 out of 27 families with the TSC2/PKD1 contiguous gene deletion syndrome, using FISH analysis for confirmation (Sampson et al. 1997a), and in 7 out of 120 TSC families with a point mutation (Rose et al. 1999), and presented more case reports (Yates et al. 1997; Kwiatkowska et al. 1999). In Chapter 3.7 we have shown that 6 out of 62 families with an identified mutation had different types of mosaicism. (Verhoef et al. 1999). Adding up the numbers of the three published case series gives a total of 20 mosaic patients in 209 families i.e. close to 10%.

In conclusion, discussing the possibility of mosaicism is an important element in the genetic counselling of many TSC families. Parental mosaicism implies that one of the (clinically unaffected) parents carries the mutated gene among his or her gametes, resulting in an increased risk of TSC in further children. Showing mosaicism in a TSC patient implies that sibs do not have an increased risk of being a carrier of the mutation. Therefore, when a

mutation in a seemingly sporadic patient with TSC is known, the parents of this patient should be offered DNA-analysis in a subsequent pregnancy, when the mutation is not detectable in DNA isolated from their white blood cells. Sibs of the patient should be offered a DNA test, in order to rule out (occult) carriership of the mutation (see also Chapter 4).

3.2
Mutational spectrum of the TSC1 gene in a cohort of 225 tuberous sclerosis complex patients; no evidence for a genotype-phenotype correlation

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Abstract

Tuberous sclerosis complex is an inherited tumour suppressor syndrome, caused by a mutation in either the TSC1 or TSC2 gene. The disease is characterised by a broad phenotypic spectrum that can include seizures, mental retardation, renal dysfunction, and dermatological abnormalities. The TSC1 gene was recently identified and has 23 exons, spanning 45 kb of genomic DNA, and encoding an 8.6 kb mRNA. After screening all 21 coding exons in our collection of 225 unrelated patients, only 29 small mutations were detected, suggesting that TSC1 mutations are underrepresented among TSC patients. Almost all TSC1 mutations were small changes leading to a truncated protein, except for a splice site mutation and two in frame deletions in exon 7 and exon 15. No clear difference was observed in the clinical phenotype of patients with an in-frame deletion or a frameshift or nonsense mutation. We found the disease causing mutation in 13% of our unrelated set of TSC patients, with more than half of the mutations clustered in exons 15 and 17 and no obvious under-representation of mutations among sporadic cases. In conclusion, we find no support for a genotype-phenotype correlation for the group of TSC1 patients compared to the overall population of TSC patients.

Keywords

Tuberous sclerosis complex, mutations, genotype-phenotype correlation

Tuberous sclerosis complex (TSC) is an autosomal dominant neurocutaneous disorder characterised by the growth of hamartomas in many tissues and organs, including brain, skin, heart, and kidney (Gomez 1988). Common neurological manifestations including seizures and mental retardation have their onset during early childhood, while cysts and angiomyolipomas in the kidney mostly become apparent during adult life. Considerable clinical variation is observed between as well as within families (Sampson et al. 1989a). At lease 60% of TSC patients represent sporadic cases, as they have unaffected parents.

Linkage analysis has shown locus heterogeneity for TSC, with one locus on chromosome 9 (Fryer et al. 1987) and a second locus on chromosome 16 (Kandt et al. 1992). About half of the large families can be linked to the TSC1 locus on chromosome 9q34 and the other half to the TSC2 locus on chromosome 16p13 (Povey et al. 1994; Janssen et al. 1994). The TSC1 and TSC2 genes were identified by positional cloning (van Slegtenhorst et al. 1997; The European Chromosome 16 Tuberous Sclerosis Consortium 1993) and there is abundant evidence that both genes act as tumour suppressor genes (Green, Smith, and Yates 1994; Carbonara et al. 1994; Henske et al. 1996a; Kobayashi et al. 1997)

The TSC2 gene consists of 41 exons, spanning 43 kb of genomic DNA (Maheswar et al. 1996). It encodes a 200 kDa protein, tuberin, which has a putative GAP activity for rab5 (Xiao et al. 1997) and rapl (Wienecke, Konig, and Declue 1995), two members of the ras superfamily of small GTPases. The mutational spectrum of TSC2 includes a number of large deletions often disrupting the PKD1 gene as well (Brook-Carter et al. 1994; Verhoef et al. 1995), but also point mutations (Vrtel et al. 1996; Wang et al. 1998; Kumar et al. 1995a, 1995b, 1997; Verhoef et al. 1998a; Au et al. 1997; Wilson et al. 1996; Au et al. 1998) and a number of missense changes (Maheswar et al. 1997). The TSC1 gene contains 23 exons and encodes an 8.6 kb mRNA. It spans 45 kb of genomic DNA and codes for hamartin, a 1164 amino acid protein of 130 kDa. Analysis of the amino acid sequence showed a potential coiled coil domain at the C-terminus but no homology to tuberin or any other known vertebrate protein was detected (van Slegtenhorst et al. 1997).

The first report describing the molecular genetic and phenotypic analysis of the TSC1 gene suggested that all mutations are small changes, that TSC1 mutations are less common in sporadically affected subjects, and that there is a reduced risk of mental retardation in TSC1 related disease (Jones et al. 1997). The goal of this study was to construct the mutational spectrum of the TSC1 gene in our collection of TSC patient samples by Southern blot and SSCP analysis. This would enable us to determine whether there is a significant difference in the detection rate between familial and sporadic cases and whether there is a genotype-phenotype correlation for the TSC1 group compared to the overall population of TSC patients.

Patients and methods

Patient selection and DNA isolation: In this study, 225 unrelated patients with tuberous sclerosis complex, diagnosed according to the criteria of Gomez (Tuberous sclerosis 1988),

were included. Eighty-two patients represented familial cases (36%) with at least one first degree relative. Three large families showed linkage to 9q34 (Janssen et al. 1994). One hundred and forty-three patients were designated sporadic cases (64%), in the absence of an apparent family history of TSC. Patients with a TSC2 mutation were excluded from the 225 cases. DNA was isolated from peripheral blood cells according to standard procedures (Miller, Dykes, and Polesky 1988). Paternity tests were performed using the Profile kit from Perkin Elmer.

Southern blotting analysis: Genomic DNA (6 µg) of 200 unrelated patients was digested with four different restriction enzymes *(Eco*RI, *Hind*III, *Pst*I and *Taq*I*)* and run on a 0.7% agarose gel. Southern blotting and hybridisation were performed using standard methods (Sambrook, Fritsch, and Maniatis 1989). Three cDNA clones were tested on the blots: a 5'RACE clone which had been amplified from a fetal brain cDNA pool (bp 24-1696) (Clontech), an RT-PCR product (bp 1616-3684) generated from fibroblast RNA, and a fetal brain cDNA clone (bp 4100-8600). The three probes cover the coding sequence as well as the 5' and 3' untranslated region of the TSC 1 gene.

SSCP analysis and DNA sequencing: Sequences of primers used for amplification of the 21 coding exons of the TSC1 gene are provided at *http://expmed.bwh.harvard.edu/projects/tsc/*. For exon 22, a new intronic forward primer was designed (5'-atactaccagcttactttccata-3'). SSCP analysis was performed according to Orita et al (Orita et al. 1989) and 2µl of the PCR product were applied to the Pharmacia GenePhor Electrophoresis system. Gels were run for 2.5 hours at 5°C and 18°C. Running conditions for two gels were 600 V, 50 mA, and 10 W. Subsequently, bands were visualised using a DNA silver staining kit (Pharmacia) in a Hoefer automated gel stainer. Variant patterns were further characterised by direct sequence analysis of the PCR products on an automated DNA sequencer (ABI 377) using the cycle sequencing dye primer kit (Perkin Elmer).

Aso hybridisation: Oligonucleotides for ASO hybridisation were designed for the mutated and normal sequence. ASO hybridisations were performed at 37°C for 30 minutes. Filters were washed to 0.3 x SSC for 10 minutes at 37°C.

Results

Screening for large abnormalities: No large insertions or deletions were identified in the TSC1 gene by Southern analysis in 200 patients. Only in one case (T2965) was an aberrant restriction pattern in a TaqI digest detected (Fig 3.1A), but no consistent change was seen with other enzymes. Comparing the genomic sequence of the TSC1 gene with the size of the extra fragment, we were able to locate the lost TaqI site in exon 15. Sequencing exon 15 of the TSC1 gene of this patient showed a C to T substitution at bp position 1719 (Fig 3.1B), resulting in the nonsense mutation R500X. The presence of this mutation was confirmed by allele specific oligonucleotide (ASO) hybridisation (Fig 3.1C).

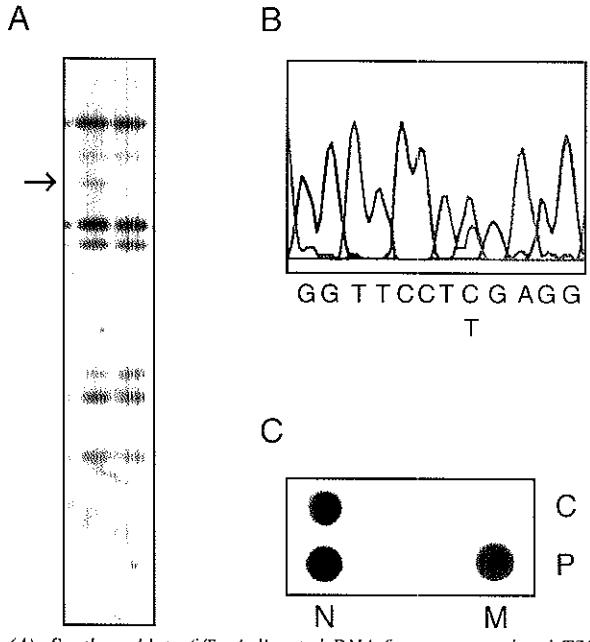
Screening for smaller mutations: Systematic SSCP analysis was undertaken to screen the 21 coding exons in the TSC 1 gene for small mutations. In total, 25 different mutations were found in 29 subjects out of the set of 225 unrelated patients (Table 3.1).

Table 3.1: Mutations identified in the TSC1 gene

Exon	Patient code	Mutation (nt substitution)	Familial/Sporadic
4	T2545	367delA	Familial
5	T1214	WI03X 529G→A)	Familial‡ ‡Sporadic†
	T1817	432-IG→A	
7	T1298	814delACT	Familial
8	T4715	944insA	Familial
	T8129	958delG	Sporadic†
9	T7806	R249X (966A→T)	Sporadic
10	T1207	1210insT	Familial
	T3945	1240delA	Familial
	T9809	Y312X (1157C→A)	Sporadic
12	T10301	1473delC	Familial
13	T1515‡	1499delT	Familial
15	T2965	R500X (1719C→T)	Sporadic
	T9886	R509X (1746C→T)§	Sporadic
	T3922	1892del23	Sporadic†
	T2067	1929delAG*§	Familial
	T5913	1978del9	Sporadic†
	T2636	2007delT*	Sporadic†
	T4124	2105delAAAG*§	Sporadic
17	T1197	R692X(2295C→T)§	Familial
	T3838		Sporadic†
	T3908		Sporadic
	T5210		Sporadic†
	T10816		Sporadic†
	T7659	2318ins28	Sporadic
	T4068	2328delCT	Sporadic
18	T2077*	R786X (2577C→T)§	Familial
20	T5406	2729delAACA	Familial
	T1295	2787delG	Familial

nt = nucleotide; *mutation reported before (van Slegtenhorst et al. 1997); †parents tested negative for the mutation; ‡family linked to chromosome 9 (Janssen et al. 1994); §recurrent mutation, also identified by other groups (van Slegtenhorst et al. 1997; Jones et al. 1997)

Figure 1A,B,C



(A): Southern blot of TaqI digested DNA from two unrelated TSC patients, hybridised with a 5' TSC1 cDNA probe (nt 24-1696). A novel 3 kb fragment is detected in the left lane, indicated by the arrow. (B): Direct sequence analysis shows the de novo nonsense mutation C→T (R500X) in a TaqI site in patient T2965. (C): ASO hybridisation of the R500X mutation in patient T2965 (P) and a control (C). N=normal allele, M=mutant allele.

All types of mutations resulting in a truncated protein have been observed: small deletions/insertions, nonsense mutations, and splice site mutations. In addition, eight different missense changes (Table 3.2) were seen in nine unrelated patients. Fourteen of the 29 mutations were small deletions, ranging from 1 to 23 bp. Three of these mutations have been reported previously (van Slegtenhorst et al. 1997). In two patients, we detected in frame deletions of 3 and 9 bp respectively. In family T1298, a 3 bp deletion in exon 7 segregated with the disease phenotype and resulted in a small amino acid change (Asp-Phe to Ile at position 198) in the protein. The grandparents, who had no signs of TSC, tested negative for the mutation. In a sporadic patient (T5913), 9 bp were deleted in exon 15, also leading to a different protein product (Cys-Lys-Ile-Pro to Ser at aa position 586). Both parents tested negative for the mutation. Biological parenthood was confirmed for both in frame deletions. All the other deletions led to a premature stop-codon. Nonsense mutations were detected in 11 cases; R692X was present in four sporadic cases and in one family. Three insertions were identified: a single base pair substitution in exon 7 and exon 10 in familial cases, and a duplication of 28 bp in exon 17 in a sporadic patient. A substitution at a splice site (bp postition 432-1) was detected in a sporadic patient, of whom the parents tested negative for the change. The most downstream mutation detected is a 1 bp deletion in exon 20 in the

middle of the coiled coil domain of hamartin. No mutations were found 3' of the coiled coil domain (amino acids 719-998).

Table 3.2 Missense and silent changes in the TSC1 gene

Exon	Code	Nucleotide change	Aa change
4	T5768	374A→C	E51D
7	T1486	789C→T	R190S*
	T4712	793T→A	L191H
8	T1524	892T→G	M224R
12	T10383	1429C→T	S403
14	T2083	1556A→G	E445*
15	T5100	2415C→T	H732Y*
	T8775		
22	T1219	3050C→T	A943*

*Confirmed polymorphism: E445, H732Y and A943 were reported before (Jones et al. 1997).
nt = nucleotide, aa = amino acid.

Missense and silent changes: Nine abnormal SSCP patterns were observed representing missense or silent changes (Table 3.2). In four cases the change was also found in the normal population. These polymorphisms were R190S (present in the unaffected parent and absent in the affected parent), E445 (allele frequency of 16% in the normal population), H732Y (allele frequency of 0.5%), and A943. E445, H732Y, and A943 have been reported before (Jones et al. 1997). None of the additional missense abnormalities have been confirmed to represent the disease causing mutation yet. E51D probably represents a polymorphism, since the amino acid change is in a conserved group. For L191H and M224R, neither parent was available for testing.

TSC1 mutations in familial and sporadic cases: Eighty-two of our 225 unrelated patients (36%) had other affected family members. Of the 29 mutations, 13 were identified in the 82 familial TSC patients (16%) and 16 in the 143 sporadic cases (11 %). Hence, we found no significant difference in detection rate between familial and sporadic cases. In half of the sporadic cases, both parents were available for analysis and tested negative for the mutation. In the other sporadic patients, DNA of both parents was not available, but there was no clinical indication of TSC disease in the family.

Clinical symptoms versus type of mutation: Patients with a mutation in the TSC1 gene were scored for the most frequent skin, brain, kidney, and heart lesions detected in TSC. A distinction was made between truncating mutations detected in TSC1 (deletion, insertion, splice site, and nonsense mutations) and the in frame deletions in exons 7 and 15 (Table 3.3).

Comparing both types of mutations, no obvious correlation could be detected between the genotype and phenotype in the TSC patients. The missense changes were left out of the analysis, because so far none of them has been confirmed to represent a disease causing mutation.

Table 3.3 Overall summary of clinical features of all patients with mutations in the TSC1 gene. A distinction has been made between patients with a stop mutation and an in frame deletion.

	Stop mutation	In frame deletion	Total
Facial angiofibroma	17/29	3/3	20/32 (63%)
Ungual fibroma	10/26	2/3	12/29 (41%)
Hypomelanotic macule	23/26	4/4	27/29 (93%)
Subependymal nodule	24/28	3/4	27/32 (84%)
Cortical tuber	10/28	2/4	12/32 (38%)
Epilepsy	23/30	2/4	25/34 (74%)
Mental retardation	13/27	1/3	14/30 (47%)
Renal cyst	4/26	0/4	4/30 (13%)
Renal angiomyolipoma	1/26	0/4	1/30 (3%)
Cardiac rhabdomyoma	5/24	1/4	6/28 (21%)

Clinical manifestations in patients with the recurrent mutation R692X: The mutation R692X was present in four sporadic TSC patients and in two patients from the same family. The clinical data of these six patients are summarised in table 3.4.

Table 3.4: Clinical features of patients with mutation R692X

	T1197a	T1197b	T3838	T3908	T5210	T10816	Total
Age(y)	56	15*	14	54	6	3	
Facial angiofibroma	+	+	-	+	-	-	3/6(50%)
Ungual fibroma	+	-	-	+	-	-	2/6 (33%)
Hypomelanotic macule	-	+	+	-	+	+	4/6 (67%)
Subependymal nodule	+	+	-	+	+	+	5/6 (83%)
Cortical tuber	-	-	-	-	+	-	1/6 (17%)
Epilepsy	+	-	+	+	+	+	5/6 (83%)
Mental retardation	-	-	?	+	-	-	1/5 (20%)
Renal cyst	-	-	?	-	-	-	0/5 (0%)
Renal angiomyolipoma	-	-	?	-	-	-	0/5 (0%)
Cardiac rhabdomyoma	-	-	+	-	+	-	2/6 (33%)

*Died aged 15 of giant cell astrocytoma.

Almost all patients have a history of epilepsy. No renal lesions were detected, but only two patients are older than 15, so these results could be biased by the later onset of cysts and angiomyolipomas. All other symptoms were scored at least once. The patients with the R692X mutation do not share an obviously similar phenotype.

Discussion

After screening the 21 coding exons of the TSC1 gene, 29 mutations were detected, all of them small changes. The only mutation detected by Southern blot analysis was the substitution of a C for T in a *TaqI* (TCGA) restriction site, resulting in a stop codon. Since four different restriction enzymes have been used to test a selection of our TSC patients on Southern blots, it is unlikely that large abnormalities disrupting the TSC 1 gene have remained undetected. Previous mutation studies in the TSC2 gene have shown a diverse mutational spectrum including large rearrangements, deletions, insertions, and nonsense and missense mutations. In the TSC2 gene, approximately 10% of the mutations detected so far were large deletions, often resulting in disruption of the neighbouring PKD1 gene as well. A possible explanation for the lack of large mutations in TSC1 may be the presence of unknown neighbouring or intragenic genes that are essential for embryonic development and survival. Although all the mutations detected in the TSC1 gene were small, we could not confirm any missense change as the disease causing mutation. Only one missense mutation in TSC1 has been reported before by Jones *et al* (personal communication), but this de novo change in the gene appeared not to be the disease causing mutation. Conversely, a number of missense mutations have been reported in the TSC2 gene (Maheswar et al. 1997). It remains to be explained why the mutational spectrum of the TSC land TSC2 genes is different. Despite the differences, most of the mutations in either TSC1 or TSC2 lead to a truncated protein, which is in concordance with a loss of function mechanism. Results obtained by interaction studies indicate that hamartin and tuberin function as a complex (van Slegtenhorst et al. 1998), which supports the phenotypic overlap observed between TSC patients with either a TSC1 or TSC2 mutation.

So far we have detected a mutation in the TSC1 gene in 13% of our unrelated TSC collection, screening all of the coding region of the gene by SSCP analysis. It is likely that this technique fails to detect all of the mutations and the promoter region has not been tested yet. We only detected the disease causing mutation in two out of three of the families which were clearly linked to chromosome 9. This number is too small to give an indication of the ratio of undetected mutations in the TSC1 gene, but we expect that for the whole group of TSC patients, the majority of the mutations will be found in the TSC2 gene. We did not observe a significantly larger number of TSC1 mutations in our familial cases than in the sporadic population, as was found in a recent study (Jones et al. 1997) although it is possible that some of our patients were misclassified as sporadic, because in only half of these cases was material from both parents available.

We found a clustering of mutations in exons 15 and 17, in which 14 out of 29 identified mutations were present. The high mutation rate in exon 15 had already been observed when the TSC1 gene was identified (van Slegtenhorst et al. 1997) and can be partially explained by the size of the exon (17% of the coding region). Furthermore, the high proportion of recurrent mutations detected in exons 15 and 17 suggests that part of these exons are particularly prone to nucleotide changes.
The truncating mutations as compared to the in frame deletions in the TSC 1 gene did not show an obvious difference in clinical phenotype in the patients, suggesting that there is no clear correlation between the nature of a TSC1 mutation and the clinical phenotype. The six patients with the recurrent mutation R692X also displayed a wide range of clinical symptoms. This is comparable to the clinical differences detected within families with TSC. The phenotypic differences in TSC patients are more likely to be caused by mechanisms such as a second hit (Green, Smith, and Yates 1994; Carbonara et al. 1994; Henske et al. 1996b) somatic mosaicism (Verhoef et al. 1995), and modifying genes. The latter has also been proposed to contribute to the complex phenotype in the comparable "monogenic" disease neurofibromatosis 1 (NF1) (Easton et al. 1993). In conclusion, we found no support for a different phenotypic spectrum in patients with a mutation in the TSC1 gene compared to the overall population of TSC patients.

The first two authors contributed equally to this work. We are grateful to the patients and their families for their participation, to the clinicians and the TSC Working Group Rotterdam for referral of the TSC patients, and to Professor.dr H. Galjaard for continuous support. This work was funded by the Dutch Organization for Scientific Research (NWO), the Dutch Praevention Fund (grant number 28-1723), and the Dutch Kidney Foundation (grant number C93.1313).

3.3
Identification of a nonsense mutation at the 5' end of the TSC2 gene in a family with a presumptive diagnosis of tuberous sclerosis complex

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Abstract

Tuberous sclerosis complex (TSC) is an autosomal dominant inherited disease with a high mutation rate. It is clinically a very variable disorder and hamartomas can occur in many different organs. TSC shows genetic heterogeneity; one gene, TSC1, is on chromosome 9q34, and the second gene, TSC2, on chromosome 16p13.3.

Clinical criteria for diagnosis have been established, but diagnosis of patients with minimal expression of the disease can be very difficult. In children the phenotype is often incomplete or not fully assessable. Hence mildly affected subjects, at risk for severely affected offspring, may remain undiagnosed.

The detection of (small) mutations in the tuberous sclerosis gene located on chromosome 16 (TSC2) has recently become possible and may be helpful in the diagnosis of ambiguous cases. To our knowledge, this is the first report of a point mutation in the TSC2 gene in a familial case of tuberous sclerosis. A nonsense mutation was detected in a family in which the father had only minor signs hinting at tuberous sclerosis. The son had multiple cardiac tumours and white patches, but full clinical investigation was impossible in this child.

This case illustrates that mutation analysis can contribute to a diagnosis of tuberous sclerosis in families with an incomplete phenotype.

Keywords

Tuberous sclerosis, TSC2 gene, nonsense mutation

Tuberous sclerosis complex (TSC) is an autosomal dominant neurocutaneous disorder characterized by hamartomas affecting multiple organ systems, including skin, kidney, brain and heart (Tuberous sclerosis 1988). The most prominent clinical signs of brain dysfunction are mental retardation and seizures. TSC is a very variable disease, with clinical manifestations ranging from very severe illness to absence of symptoms. Also, considerable variation is observed within families (Smalley, Burger, and Smith 1994). The prevalence of tuberous sclerosis has been reported to be 1:6.000 to 1:10.000 (Osborne, Fryer, and Webb 1991). However, considering the variability of the disease, it has been suggested that the prevalence is underestimated (Brook-Carter et al. 1994).

Locus heterogeneity has been shown by linkage analysis (Sampson et al. 1989b; Haines et al. 1991; Povey et al. 1991; Northrup et al. 1992) One locus for tuberous sclerosis, TSC1, has been assigned to chromosome 9q34 (Fryer et al. 1987), while the second locus, TSC2, is on chromosome 16p13.3 (Kandt et al. 1992). Approximately half of the families can be linked to either locus. Sporadic patients represent about half of the total tuberous sclerosis patients population. Mutations are expected in equal proportion in either the TSC1 or TSC2 gene. No evidence exists for clinical differences in severity, nor in range of symptoms, between chromosome 9 and chromosome 16 linked families (Kwiatkowski and Short 1994). In one report there is a suggestion of a mild physical phenotype with behavioral problems in a chromosome 16 linked family (Smalley, Burger, and Smith 1994).

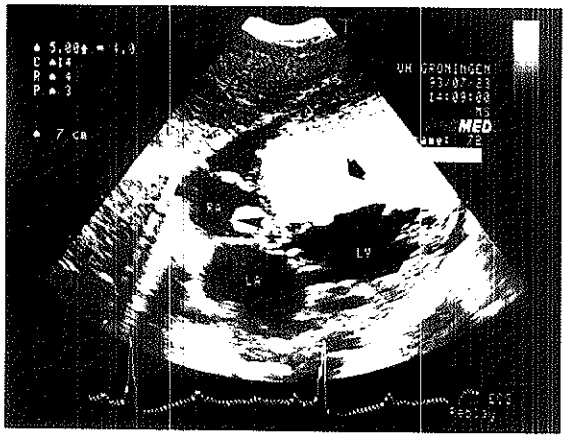
Recently, the TSC2 gene has been cloned (The European Chromosome 16 Tuberous Sclerosis Consortium 1993). Taking into account the loss of heterozygozity of the TSC2 containing region observed in renal angiomyolipoma, cardiac rhabdomyoma, and giant cell astrocytoma of tuberous sclerosis patients, it has been suggested that the TSC2 gene acts as a tumour suppressor gene (Green, Smith, and Yates 1994).

In tuberous sclerosis, rearrangements have been identified in 4% of the patients using pulsed field gel electrophoresis and Southern blotting (Brook-Carter et al. 1994; The European Chromosome 16 Tuberous Sclerosis Consortium 1993; Verhoef et al. 1995) No point mutations, detectable by single strand conformation polymorphism (SSCP) and related techniques, have been reported. Mutation analysis for TSC might be compared to that of neurofibromatosis type 1 (NF1), another neurocutaneous disorder caused by mutations in a tumour suppressor gene (Cawthon et al. 1990; Upadhyaya, Shaw, and Harper 1994) To date, in the NF1 gene no hot spot for mutations has been published. In this article we report a point mutation in the TSC2 gene, present in two family members, a father and his son. Familial tuberous sclerosis was suspected but could not be proven on clinical grounds.

Case Reports

At 20 weeks of her first pregnancy the mother of the index case was referred to a specialised unit for prenatal diagnosis because of fetal bradycardia and arrhythmia. At 24 weeks' gestation an intracardiac mass suspected of being a rhabdomyoma was detected by fetal ultrasound evaluation. The likely diagnosis of tuberous sclerosis was made.

Figure 3.2



Echocardiographic subcostal four chamber view showing the large septal tumour (big arrow) and a smaller tumour (small arrow) in the atrioventricular region. LA = left atrium, LV = left ventricle, RA = right atrium.

Delivery of a boy, weighing 2500 grams, was uncomplicated at 39 weeks. The postnatal ECG revealed an intermittent second and third degree atrioventricular block. The echocardiogram showed a large multilobulated cardiac tumour and two additional smaller tumours, one of which was situated in the region of the atrioventricular node (Fig 3.2). Echograpy of the skull was normal at 1 month of age. Investigation of retina and echography of liver and kidneys showed no abnormalities. At 3 months of age a hypomelanotic macule, 25 x 15 mm, was noted on the buttock using Woods light. Recent evaluation at the age of 18 months showed the intracardiac tumours to be smaller. Although cardiac function was still impaired, atrioventricular conduction had normalised. Another hypomelanotic patch was seen on the leg, 15x15mm. Psychomotor development was apparently normal. CT scan was postponed in the index case because of the risk of complications of complete anaesthesia. The features elicited in the child would allow a presumptive but not a definite diagnosis of TSC, according to the criterial of Gomez (Table 3.5), though the diagnositic criteria of Webb and Osborne would be fulfilled (Webb and Osborne 1995).

The proband's father was evaluated at 30 years of age. CT scan of the brain, echography of the heart, and investigation of skin (Woods light) and retina were normal. Echography of the kidney showed slightly increased echodensity with unilateral multiple echodensities 1 mm in diameter, located either in the capsula or in the peripheral cortex. These echodensities werd non-specific, unlike renal angiomyolipoma, the characterisitic renal lesion in TSC, which is generally located in the parenchyma in combination with renal cysts. On all tooth surfaces pit shaped enamel defects were seen, corresponding to the dental pits described in patients with tuberous sclerosis (Hoff et al. 1975). In addition two gingiva fibromas were found (Fig. 3.3). Thus, the father shows two "suspect" oral symptoms (Table 3.5).

Figure 3.3 The tooth surfaces of the father (II-3) with pit shaped enamel defects and two gingiva fibromas.

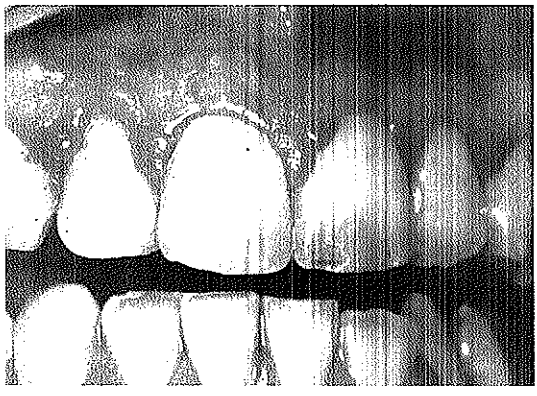
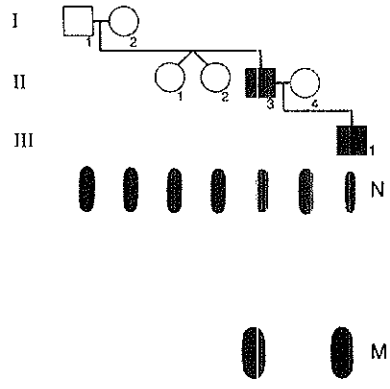


Figure 3.4 Allele specific oligonucleotide hybridization for the detection of the K12X mutation



N = normal allele. M = the mutated allele. Note that the normal alleles are about equally reduced intensity in the affected family members II-3 and III-1.

In summary, from the combination of signs in the father and son are it is very likely that they both have TSC. All first degree relatives of father and son were screened for signs of tuberous sclerosis, but no abnormalities were detected on full clinical evaluation.

Methods

Medical examination: All first degree relatives of III-1 and II-3 were screened for signs of tuberous sclerosis. Physical examination, dermatological examination with Woods lamp, a CT-scan of the brain, X-rays of both hands and feet and echographical examination of heart and kidneys were performed in all family members, including the father of the proband, and found normal.

Mutation analysis: In principle all patients included in mutation analysis fulfilled the diagnostic criteria of Gomez (1991), unless circumstantial evidence, like in this case, was highly suggestive of tuberous sclerosis or related disease. Genomic DNA was extracted from peripheral blood leucocytes by the salting out procedure (Miller, Dykes, and Polesky 1988).

Structure of the TSC2 gene: A genomic phage library of the TSC2 region was prepared. Exon-intron boundaries were determined by sequencing with vector primers (Maheswar et al. manuscript submitted).

Table 3.5 Clinical or imaging features for the diagnosis of TSC (adapted from Gomez 1991)

Diagnostic features+			
Organ	Definitive signs	Presumptive signs	Suspect signs
CNS	Cortical tubers Subependymal nodules		Infantile spasms Seizures
Retina	Hamartomas	Hamartoma (single)	
Skin	Facial angiofibromas Ungual fibroma Fibrous forehead plaque	Confetti-like spots	Hypomelanotic maculas
Kidneys	Multiple angiomyolipomas	Angiomyolipoma	Cysts
Heart		Multiple rhabdomyomas	Rhabdomyoma
Teeth			Enamel pits
Gingiva			Fibromas
Bones			Cysts Osteoma thickening
Symptoms at the time of clinical assessment in the K12X family			
Person	Definitive signs	Presumptive signs	Suspect signs
Grandmother	-*	-	-
Father	-	-	Enamel pits Gingiva fibromas
Son	-#	Multiple rhabdomyomas	Hypomelanotic maculas

+ Only the more frequent signs that are normally screened for in relatives of TSC patients are mentioned.
* Diagnostic signs mentioned in top part of table excluded by adequate clinical examination.
# CT scan postponed in the index case.

Exon amplification: SSCP analysis was performed according to the method described by Orita et al. (Orita et al. 1989). Primers for amplification of exon 1 of the TSC2 gene were as follows: forward 5'-cagaggtgttgctcagatgtccc-3', reverse 5'-atttccctctagcctagcaaaga-3'. The length of the normal PCR product is 256 base pairs. PCR conditions for 10 µl volume were 1 mmol MgCl2; 0.5 mmol spermidine; 1 pmol of each primer; 200 µmol mix of dATP, dGTP, dTTP; 2.5 µmol dCTP; 0.06 µl ^{32P}dCTP (10mCi/ml) (ICN) and 0.2 U of Taq polymerase (Gibco/BRL). Thermal cycling conditions were 6 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 90 seconds at 72°C, with a final elongation of 6

minutes at 72°C. Electrophoresis was performed on a 6% polyacrylamide gel with 10% glycerol. Running time was 14 hours, 5 Watts, both at 4°C and at room temperature.

Sequence analysis: For sequencing of PCR products, 100 µl of PCR product was purified and concentrated on microcon 30 (Amicon). The double stranded DNA cycle sequencing system (Gibco/BRL) was used to sequence PCR fragments directly: 6 minutes at 94°C followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 60 seconds at 72°C, and 6 minutes at 72°C in the last cycle. Sequencing of both strands was performed with the primers used for the SSCP analysis.

ASO hybridization: For the ASO hybridization the sequence of the "normal" oligonucleotide was 5'-CTTGAAGGAGAAGTTTA-3' and for the "mutant" oligonucleotide 5'-CTTGTAGGAGAAGTTTA-3'. Hybridization was performed at 37.5oC for 30 minutes. Filters were washed until 0.3 SSC for 10 minutes at 37.5oC.

Marker analysis: Markers 3'HVR, KG8 and 16AC2.5 (D16S291) were analyzed according to standard procedures (Current protocols in human genetics 1994).

Results

Mutation analysis of the TSC2 gene in TSC patients has been performed using Pulsed Field Gel Electrophoresis (PFGE) and Southern blotting analysis, resulting in the detection of a number of large deletions (Brook-Carter et al. 1994; The European Chromosome 16 Tuberous Sclerosis Consortium 1993; Verhoef et al. 1995) However, the majority of the TSC2 gene defects are expected to be small mutations. To facilitate the detection of such mutations, the genomic organization of the TSC2 gene was determined (Maheswar et al. manuscript submitted) and exon specific primers were designed for single strand confirmation polymorphism (SSCP) analysis.

DNA from 116 unrelated TSC patients was analyzed to detect mutations in the first exon (nucleotides 1-156 of the published TSC2 cDNA sequence) (The European Chromosome 16 Tuberous Sclerosis Consortium 1993). In one case an additional band was observed. Subsequent sequencing showed an A→T transition at nucleotide position 52, resulting in a change of lysine (AAG) to a stop codon (TAG) at amino acid position 12 (K12X). Allele specific oligonucleotide hybridization (ASO) was performed on DNA from all family members (Fig. 3.4). The K12X mutation was also present in the proband's father. The other family members, including the twin-sisters of the father and their parents, were homozygous for the normal allele. To trace the origin of the mutated allele, linkage analysis with the VNTR probe 3'HVR, located distally to the TSC2 gene, and polymorphic CA-repeat markers KG8 and 16AC2.5 (D16S291) was undertaken. KG8, the closest marker just proximal to the TSC2 gene, was not informative. 16AC2.5 is positioned about 200 kb proximal of the TSC2 gene (Janssen et al. 1994). Both informative flanking markers proved the mutated chromosome to be of grandmaternal origin. Both twin-sibs had received the other grandmaternal chromosome 16 (data not shown).

Discussion

Tuberous sclerosis is a clinically and genetically heterogeneous disease. Half of the patients are expected to have a mutation in the TSC2 gene. From our file of 119 patients, three patients had large deletions in the gene (The European Chromosome 16 Tuberous Sclerosis Consortium 1993; Verhoef et al. 1995) On the hypothesis of an equal distribution of mutations across the TSC2 gene, which contains 41 exons (Maheswar et al., manuscript submitted), one or two mutations per exon per 100 patients may be expected. SSCP analysis of the first exon of the TSC2 gene resulted in the identification of the first point mutation. No other mutations were detected in the first exon.

The mutation was found only in the father and in the son. Analysis with flanking polymorphic markers showed that the mutated allele is of grandmaternal origin. After complete clinical examination, the grandmother showed no signs of tuberous sclerosis. Germ line mosaicism in the grandmother or a de novo mutation in the father in the early stages of postzygotic cell division has occurred, since the mutation could not be identified in DNA from leucocytes of the grandmother. The possibility of germ line mosaicism in the grandmother prompted the analysis of the father's sisters, who were shown to be unaffected, both by mutation analysis and by marker analysis. Recently, we described a case of somatic mosaicism in TSC, based on the identification of a familial mutation (Verhoef et al. 1995). From our analysis there is no suggestion that the father is a somatic mosaic for the mutation although the possibility of a very high grade (>90%) mosaicism cannot be excluded on the basis of the ASO result (Fig. 3.4). The father showed signs that make him a tuberous sclerosis patient if the diagnosis is certain in the child, given the criterium of the affected first degree relative (Gomez 1991). The detection of the mutation allowed the suspected affected status in the father to be confirmed, thus making an important contribution to the diagnosis of familial TSC and genetic counseling in this family.

The K12X mutation leads to a truncated peptide of 11 amino acids in length. In the TSC2 gene an alternative downstream ATG codon is present at amino acid position 50. The nucleotide sequence of this alternative site is partially consistent with the criteria proposed by Kozak (Kozak 1987). It is feasible that transcription could start at this position resulting in a protein with a lower molecular weight and lacking part of the N-terminus of the protein. None of the affected family members requires medical treatment for epilepsy, has severe mental impairment, or proven renal involvement. These observations may support the hypothesis of the usage of an alternative ATG codon resulting in an altered, partially functional tuberin. It is probable that the amount of alternative product and its activity would differ from the activity of the normal tuberin protein. Since no antisera directed against tuberin are available as yet, this hypothesis has not been tested.

Mosaicism could account for a difference in phenotype in this family, but the father is at the most a high-grade mosaic. Another partial explanation for the difference in phenotype between proband and father may be found in the different ages at which different signs of

tuberous sclerosis are most apparent. Cardiac manifestations have been reported to decrease in both clinical importance and echocardiographic detectability, especially in the first years of life (Watson 1991). It is thought that the tumours either regress or are incorporated in the cardiac muscle wall. There is no clinical history of neonatal or infantile heart disease in the father. Dental pits have been reported to be poorly visible in primary teeth. Gingiva fibromas often appear only at puberty. The child may still develop oral signs later in life.

With regard to genetic counselling of this family it has to be noted that the occurrence of a full, possibly severe, phenotype of tuberous sclerosis cannot be excluded for the child yet or for future generations. However, it is also possible that in this family the observed genotype gives a consistent phenotype. More mutations in clinically well delineated families are needed to decide on the extremely important issue of genotype-phenotype correlations. Once a definitive diagnostic test becomes available, and the majority of patients can be detected by DNA analysis, re-evaluation of the diagnostic criteria will be needed.

Our results prove the diagnosis of tuberous sclerosis in this family by means of mutation detection. We recommend molecular analysis in families in which a diagnosis of tuberous sclerosis is considered, even if the diagnosis cannot be made on clinical criteria alone.

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Note:

After submission of the article, the first point mutation in the tuberin gene was reported by A Kumar et al.(Kumar et al. 1995a).

3.4
Identification of a large insertion and two novel point mutations (3671del8 and S1221X) in tuberous sclerosis complex (TSC) patients

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Human mutation, Mutations in brief #119 (1997) Online.
Website http://journals.wiley.com/humanmutation.

Abstract

Important symptoms of tuberous sclerosis complex (TSC), an autosomal dominant disorder, are hamartomata in several organs, mental retardation and epilepsy. Either one of two loci can be involved (TSC1 and TSC2), of which the TSC2 gene has been cloned. To date, only 35 mutations in the TSC2 gene have been described ranging from large deletions to point mutations. Southern blot analysis using cDNA clones of the TSC2 gene was performed on a cohort of 160 unrelated TSC patients and revealed a 10 kb insertion. The insertion was also present in DNA of the affected father. Both patients showed renal angiomyolipoma, hypomelanotic macules and epilepsy. SSCP analysis of exons 1, 2, 3, 9, 12, 14, 30a and 36 identified two mutations in exon 30a: 3671 del8 and S1221X. Symptoms of the sporadic patient with the 3671del8 mutation are cortical tubers, subependymal nodules, facial angiofibroma, ungual fibroma, renal angiomyolipoma, hypomelanotic macules, epilepsy and mental retardation. Clinical symptoms of the patient with the S1221X mutation are facial angiofibroma, ungual fibroma, hypomelanotic macules, epilepsy and mental retardation. His parents were negative for the S1221X mutation, although a germline mosaicism can not be excluded. Besides the previously described polymorphism 1596C→T, two rare variants were observed, a substitution of C→T at position 1294 and at position 1299 C→A.

Key Words:
TSC: Tuberous sclerosis complex.
TSC 1 : McKusick 191100, .GDB.
TSC 2: McKusick 191092, .GDB.

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder with an incidence of 1: 6,000 – 10,000, showing both clinical variability and locus heterogeneity. Important signs and symptoms of TSC are mental retardation, epilepsy, facial angiofibromas, subependymal nodules and cortical tubers in the brain and the occurrence of hamartomata in many organs, e.g. retinal hamartoma, renal angiomyolipoma and cardiac rhabdomyoma (Gomez 1991). Linkage analysis indicated the involvement of either the locus on chromosome 9 (TSC1) or on chromosome 16 (TSC2) (Janssen et al. 1994). About half of the TSC patients are considered to be sporadic cases, caused by new mutations. The TSC2 gene, consisting of 41 exons, has been cloned, making mutation analysis in TSC patients feasible (The European Chromosome 16 Tuberous Sclerosis Consortium 1993; Maheswar et al. 1996) A spectrum of mutations ranging from large deletions (20) to point (15) mutations have been identified (The European Chromosome 16 Tuberous Sclerosis Consortium 1993; Brook-Carter et al. 1994; Verhoef et al. 1995; Kumar et al. 1995b; Kumar et al. 1995a; Vrtel et al. 1996; Wilson et al. 1996; Au et al. 1997; Kumar et al. 1997; Verhoef et al. 1998b).

In this article mutation analysis in a cohort of 160 patients is presented using a combination of Southern blot and SSCP analysis. Southern blots of genomic DNA were hybridised with the TSC2 specific cDNA clones 4B2 (HindIII digestion), 4.9E0.7 (HindIII digestion) and 1A1 (EcoRI digestion). The localization of the probes is depicted in Figure 3.5A. SSCP analysis of exons 1,2,3,9,12,14,30a and 36 was performed as described previously (Vrtel et al. 1996). Briefly, after PCR electrophoresis for the SSCP analysis was performed on a 6% polyacrylamide gel containing 4% glycerol. Duplicate gels were run for 14 hours (5W) at 4°C and at room temperature. Primer sequences used for SSCP and sequence reaction are given in Table 3.6 (exon numbering is based on the published cDNA sequence; therefore the alternatively spliced exon numbered 31 by Maheshwar et al. (1996) is exon 30b).

Southern analysis

10 *kb insertion (+ deletion?):* Using the probe 4B2 on HindIII digested genomic DNA of patient T1268 the 14 kb HindIII fragment appeared to be reduced in intensity compared to normal control samples, whereas the intensity of the 18 kb fragment was normal. In addition, two aberrant fragments of 13 and 10 kb were present (data not shown). Hybridisation of EcoRI digested genomic DNA with the probe 4B2 showed the 9 kb and 3 kb fragments with normal intensity, reduced intensity of the 18 kb EcoRI fragment and two aberrant fragments of 20 and 12 kb (Fig 3.5B). The 20 kb fragment was also identified by the 4.9E0.7 probe (data not shown). No abnormal patterns were identified using the 1A1 probe on either EcoRI or HindIII digested DNA. Based on the intensities of the normal and aberrant fragments a duplication of TSC2 sequences is very unlikely. Therefore, this aberrant pattern is the result of an insertion of 10 kb in the middle of the genomic region covering the TSC2 gene, although the combination of an insertion with a deletion can not be ruled out. The abnormality was also present in DNA of the father of the patient. Since no material of the paternal grandparents was available, the parental origin of the mutation could not be investigated. The

affected father has had severe renal problems with hematuria. He has epilepsy and is of normal intelligence. His son has multiple manifestations of TSC, is mildly mentally retarded and also has seizures (Table 3.7).

SSCP analysis

SSCP analysis of exon 12 resulted in the identification of 2 different abnormal patterns in 3 out of 160 unrelated families. Direct sequence analysis showed the presence of the substitution of C→T at position 1294 (one family) and at position 1299 C→A (two families). These changes do not result in amino-acid substitution, indicating that they are rare variants.
The recently described polymorphism 1596C→T in exon 14 (Wilson et al. 1996) was also identified in our SSCP analysis. Using ASO hybridisation, the frequency of this polymorphism was determined to be 5.6% (18/320 alleles) in our TSC population and 8.1% (15/186 alleles) in a normal control population. This frequency seems to be higher than reported by Wilson et al (1/60 alleles) in a different population.
3671 del8 mutation: SSCP analysis of exon 30a showed an abnormal pattern in patient T1756, who was referred to us as a sporadic case. Direct sequence analysis identified an 8 bp deletion at position 3671, resulting in a frameshift and a stopcodon at position 1230. Using Allele Specific Oligonucleotide (ASO) hybridisation the observed sequence abnormality was confirmed in DNA of the index patient (Figure 3.5C). Since no material of the parents was available, the origin of the mutation could not be established. This now 60-year old male is institutionalised in a specialised epilepsy clinic. He shows several signs of TSC, including facial angiofibroma, renal angiomyolipoma and hypomelanotic macules (Table 3.7). He developed a colon carcinoma, which was classified as an adenocarcinoma. Using the ASO hybridisation analysis both the wild type and mutant allele were present in the tumour (Figure 3.5C), making the involvement of the TSC2 gene in the etiology of this tumour unlikely.
S1221X mutation: In another sporadic patient (T3998) a different abnormal SSCP pattern was observed in exon 30a. Sequence analysis identified a C→A substitution at position 3680, resulting in a stopcodon (S1221X). The mutation could not be detected in genomic DNA isolated from venous blood of the parents using ASO hybridization (Figure 3.5D). This result does not exclude the possibility of germline mosaicism in one of the parents. The patient is severely retarded, with epilepsy. Although he did not undergo extreme clinical examination, his diagnosis is certain on the basis of Vogt's triad plus multiple ungual fibromas. He had an operation for diaphragmatic hernia and at that time the kidneys were macroscopically normal (Table 3.7).

Based on linkage analysis it was hypothesized that about half of the TSC patients will have a mutation in the TSC2 gene. Using the exon based SSCP analysis, about 20% of the TSC2 coding region has been tested, which resulted in the identification of 5 mutations (Vrtel et al. 1996; Verhoef et al. 1998a) and this report. To date, no hot spot for mutations in the TSC2 gene has been identified. Therefore, extrapolating our SSCP results to the complete TSC2

gene and combining these data with our previously reported mutations (The European Chromosome 16 Tuberous Sclerosis Consortium 1993; Verhoef et al. 1995) in about 20% of all our TSC families the mutation can be identified. If 50% of TSC patients would have a TSC2 mutation, SSCP analysis would be expected to detect a mutation in 30-40% of the cases, under the assumption that SSCP detects 70-80% of mutations in genomic DNA (Cotton 1993). After Southern blot analysis, Wilson et al used RT-PCR products to identify the mutations with SSCP analysis and were able to detect mutations in 30% of their patients. This efficiency is somewhat higher than in our studies, which might be influenced by a higher percentage of chromosome 16 linked families in the study of Wilson et al. (2/160 and 2/30, respectively). Since the total number of chromosome 16 linked families included in our mutation analysis is low, the low number of mutations detected might also reflect the difference between familial and sporadic TSC patients with respect to the TSC gene involved.

Table 3.6: Primer sequences used in SSCP analyses.

Exon	Primer Sequence	Position to Exon	Size PCR Product (bp)
1	F: 5'-cagaggtgttgctcagatgtccc-3'	-74*	253
	R: 5'-atttccctctagcctagcaaaga-3'	+41	
2	F: 5'-tgcagttaaggagaccgtgg-3'	-61	197
	R: 5'-ctgaatagtctacgtgcctct-3'	+49	
3	F: 5'-gccagggttcttggagagcac-3'	-53	221
	R: 5'-acaggacagtcagtgggcagc-3'	+57	
9	F: 5'-gggacagggccctgctcacatt-3'	-47	212
	R: 5'-cgttcctgccagctcactgca-3'	+38	
12	F: 5'-gaggggcaacaccggctcttc-3'	-63	187
	R: 5'-agggccaggctccaggtgcca-3'	+30	
14	F: 5'-cgctcattggcctcccttg-3'	-30	231
	R: 5'-gagctctggcacgctagcc-3'	+45	
30a	F: 5'-agatgggtaaggggaggtac-3'	-56	298
	R: 5'-ggagcagagcccgtgccaa-3	+38	
36	F: 5'-cagcactggccccacaaaccc-3'	-43	249
	R: 5'-tgccaccaacccggacacagc-3'	+76	249

* Position to ATG

Table 3.7: Clinical data of the patients with the identified mutation in the TSC2 gene.

Diagnostic category (Gomez, 1991)	Symptom/sign	T1268 ins ± 10 kb father 1948*	T1268 ins ± 10 kb son 1989	T1756 3671 del8 exon 30a male 1936	T3998 S1221X exon 30a male 1959
Definitive	Cortical tubers		+	+	
	Subependymal nodules		+	+	
	Facial angiofibroma	+	-	+	+
	Ungual fibroma	+	-	+	+
Presumptive	Renal angiomyolipoma	+	+	+	
	Renal cysts	+	-		
	Retinal hamartoma		-		
Suspect	Cardiac rhabdomyoma		+	-	
	Hypomelanotic macules	+	+	+	+
	Buccal fibroma	+			
	Multiple dental pits	+			
	Epilepsy	+	+	+	+
Aspecific	Mental retardation	-	+	+	+
	Diaphragmatic hernia				+
	Colon carcinoma			+	
	Macrocephaly		+		
	Hypotonia		+		

*: year of birth, +: present, -: absent

T3998: This patient is a severely retarded institutionalised patient, who is reasonably healthy apart from his epilepsy and thus not thoroughly investigated clinically. His diagnosis, however, is certain on the basis of Vogt's triad plus multiple ungual fibromas. He had an operation for his diaphragmatic hernia, at which time the kidneys were inspected and macroscopically normal.

T1756: This now 60-year old male is institutionalised, in an specialised epilepsy clinic. He developed a colon carcinoma at the age of over 50 years. The colon carcinoma was classified as an adenocarcinoma.

T1268: The affected father, with normal intelligence and a normal job, has severe renal problems, without hypertension. He has to undergo bladder wash-outs about every two years

to remove blot clots from the bladder. He has had persistent haematuria for years. He is free of epilepsy with a low dose of antiepileptic drugs.
The son has multiple manifestations and is mildly mentally retarded. He has had no seizures for about 2.5 years with diphantoin and depakine.

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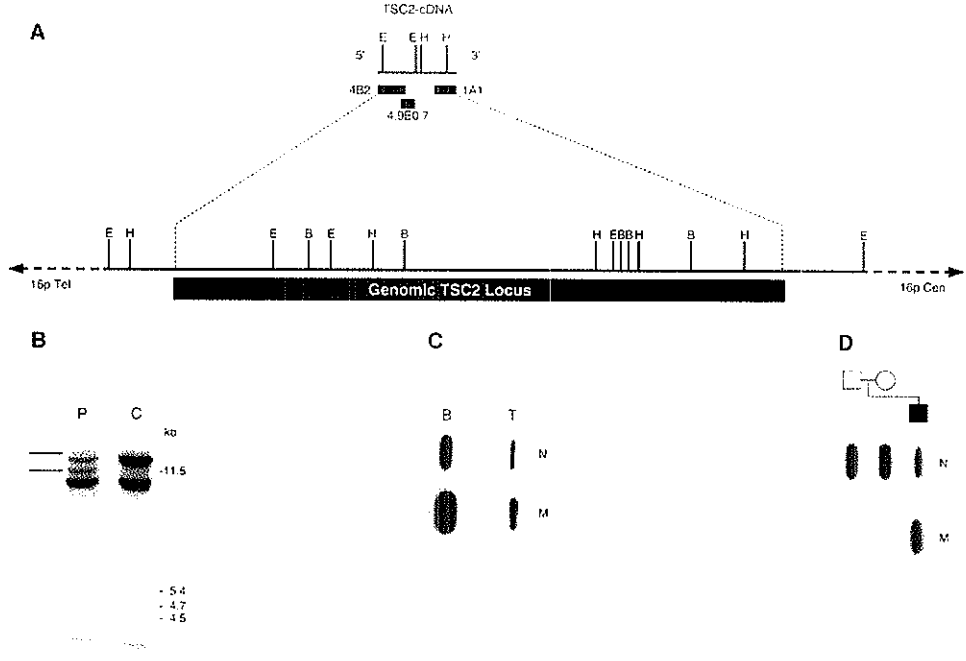
Figure 3.5: Restriction map of the TSC2 gene. In the upper part the cDNA probes 482, 4.9E0.7 and 1A1 used in the Southern blot analysis are depicted. B=BamHI; E=EcoRI; H=HindIII.



(B) Southern blot analysis of EcoRI digested genomic DNA of patient T1268 and hybridised with the 4B2 probe. P=Patient T1268, C=Control. The arrows indicate the aberrant fragments.
(C) Allele Specific Oligonucleotide (ASO) hybridisation analysis of the 3671del8 mutation in DNA isolated from blood (B) and from the adenocarcinoma (T) of patient T1756. N=normal allele, M=mutant allele.
(D) ASO hybridisation analysis of the S1221X mutation in family T3998. N=normal allele, M=mutant allele.

3.5
Recurrent Mutation 4882delTT in The GAP-related domain of the tuberous sclerosis TSC2 gene

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Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant neurocutaneous disorder with a very variable clinical picture. It is characterised by hamartomas in skin, central nervous system and in any organ system except in skeletal muscle (Tuberous sclerosis 1988). The most important clinical signs are epilepsy, mental retardation, facial angiofibromas, hypomelanotic patches and renal angiomyolipomas. The estimated prevalence of the disease is between 1:6000 and 1:10.000 live births (Osborne, Fryer, and Webb 1991).

Locus heterogeneity has been established for TSC, with one gene (TSC1) on chromosome 9q34 and a second gene (TSC2) on chromosome 16p13 (Janssen et al. 1994). The TSC1 gene has not been cloned, while the TSC2 gene has been identified (The European Chromosome 16 Tuberous Sclerosis Consortium 1993). About one-half of the patients are thought to have new mutations. Based on linkage data, it is expected that about 50% of the patients have a mutation in the TSC2 gene. The characterisation of the TSC2 gene made mutation analysis feasible in TSC patients.

The TSC2 gene consists of 41 exons (Maheswar et al. 1996), of which exon 36 contains the region of homology to the GTPase-activating protein GAP3 (numbering of exons was based on the cDNA sequence published by The European Chromosome 16 Tuberous Sclerosis Consortium, 1993). The alternatively spliced exon 31 (Maheswar et al. 1996) was numbered as exon 30b. Recently, it was shown that tuberin indeed acts as a GTPase-activating protein (Wienecke, Konig, and Declue 1995). Therefore, mutations in this region may help study gene function. Several large deletions in TSC2 have been detected by Pulsed Field Gel Electrophoresis and Southern blot analysis (The European Chromosome 16 Tuberous Sclerosis Consortium 1993; Brook-Carter et al. 1994; Verhoef et al. 1995) A small number of point mutations, detected by SSCP analysis, have been reported (Kumar et al. 1995b; Kumar et al. 1995a; Wilson et al. 1996; Vrtel et al. 1996)This paper describes the first recurrent mutation in the TSC2 gene. The mutation is located in exon 36, the exon containing the region of homology to the GTPase-activating protein GAP3 (The European Chromosome 16 Tuberous Sclerosis Consortium 1993).

Methods

The study included 140 unrelated TSC patients. They were screened by Southern blot analysis using TSC2-specific cDNA probes and FISH techniques for the detection of large mutations in TSC2 and showed no abnormalities; they were subsequently analysed by using Single Strand Conformation Polymorphism (SSCP) analysis by the method of (Orita et al. 1989). Primer sequences to perform the PCR of exon 36 were: forward 5'-cagcactggccccacaaaccc-3', reverse 5'-tgccaccaacccggacacagc-3'. The length of the normal polymerase chain reaction (PCR) product was 250 base pairs (bp). PCR conditions for 10 µl reaction mixture were: 60 ng DNA; 1 mM MgCl2, 200µM each of dATP, dGTP, dTTP; 2.5µM dCTP; 1 pmol of forward and reverse primer; 0.2 U Taq polymerase (Gibco/BRL); 0.5 µl 1%W1; 1 µl DMSO, 0.06 µl α32P-dCTP(10 mCi/ml;ICN). Thermal cycling conditions were 10 min at 94°C, followed by 25 cycles of 30 sec

at 94°C, 60 sec at 62°C, 90 sec at 72°C, with a final elongation of 20 min at 72°C. Electrophoresis for the SSCP was performed on a 6% polyacrylamide gel containing 4% glycerol 4.5 hours, 25 W, at both 4°C and room temperature.
For the Allele Specific Oligonucleotide hybridisation (ASO), the sequence of the "normal" oligonucleotide was 5'-CAACGACTTTGTGTCCA-3' and for the "mutant" 5'-CAACGACTGTGTCCATT-3'. Hybridisation was performed at 44°C for 60 minutes. Filters were washed in 3x SSC (20 min.), 1x SSC (10 min.) with a final step at 0.3x SSC for 2 min at 44°C.

Results and discussion

Two patients showed the same aberrant pattern after SSCP analysis of exon 36 of the TSC2 gene (not shown). Direct sequencing of the PCR product of exon 36 with the same primers as for the SSCP analysis, revealed an identical mutation: a deletion of two nucleotides TT at nucleotide position 4882-4883. The 4882delTT mutation was confirmed by (ASO) hybridisation (Fig 3.4). ASO performed on all 140 patients showed no other patients with the mutation.

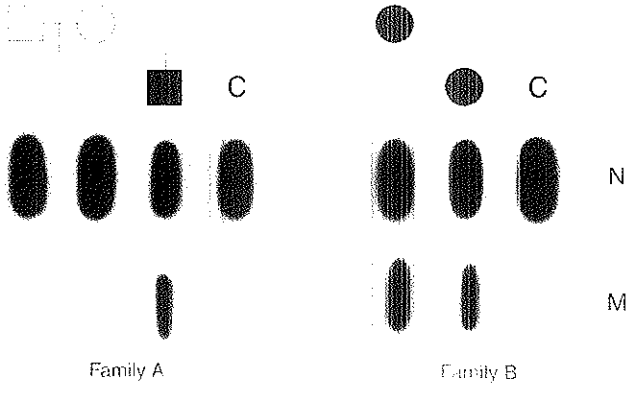
Table 3.8: Clinical data of the patients with the 4882delTT deletion

Diagnostic Category (Gomez 1991)	Symptom/sign	family A	family B	
		boy age14y	girl age18y	mother age40y
Definitive	Subependymal nodules	+	+	+
	Facial angiofibroma	+	+	+
	Ungual fibroma	+	+	+
	Fibrous plaques	+	+	+
Presumptive	Renal angiomyolipoma	-	+	+
	Renal cysts	-	-	-
	Retinal hamartoma	-	+	-
Suspect	Cardiac rhabdomyoma	-	+	+
	Hypomelanotic macules	+	+	+
	Shagreen patches	-	+	+
	Buccal fibroma	+	+	n.d.
	Multiple dental pits	+	n.d.	n.d.
	Epilepsy	+/-	+	+
Aspecific	Mental retardation	-	-	-

+ = present, - = absent, +/- = present under 2 years, later absent, n.d.= not determined

The clinical data are presented in Table 3.8. In family A, of Dutch origin, the index patient was diagnosed as having TSC at the age of 4 years. Both parents were fully investigated for signs of TSC and, apart from two hypomelanotic macules in the father, the parents did not show any suggestion of the disease. ASO hybridisation showed that the 4882delTT mutation was absent in DNA isolated from leukocytes from the parents (Fig 3.6). This indicates that the mutation has arisen *de novo* either in one of the parents or postzygotically in the patient.
In family B, of Polish descent, mutation analysis was performed initially using DNA of the affected daughter (Fig 3.6). Subsequent ASO analysis of the available family members showed that the 4882delTT mutation was also present in DNA of the clinically affected mother. The maternal grandparents were not available for DNA analysis; therefore the parental origin of the 4882delTT mutation can not be traced in this family.

Figure 3.6: Identification of the 4882delTT mutation in genomic DNA using allele specific oligonucleotide hybridization.



N = normal allele, M = mutated allele, C = negative control.

The 4882delTT mutation leads to a frameshift causing a stop codon (TGA) at amino acid position 1628, presumably leading to deletion of the C-terminal part of the tuberin protein and loss of the functional GTPase-activating domain. Since in only two out of 140 patients tested a mutation in the GAP-related domain was identified, it is clear that there is no hot spot for mutations in this domain.
All three 4882delTT patients have several of the characteristic signs of the disease although no specific sub-phenotype could be established. Extensive skin involvement and computer tomography (CT) scan abnormalities were seen in all three patients, without coexisting mental retardation. The boy in family A had episodes of "febrile convulsions", the last one at the age of almost two years, but has not shown any signs of epilepsy since, both patients in family B have epilepsy. For the first time the same mutation was observed in two tuberous sclerosis families:

one familial case, one sporadic case. A possible relationship between the Dutch and Polish families was investigated by using high polymorphic repeat markers (data not shown). These analyses showed that the families are not related, confirmed the family structures, and ruled out the possibility of sample switches. Therefore, the mutation occurred as two separate events and is a true recurrent mutation.

Acknowledgments

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3.6
Somatic mosaicism and clinical variation in tuberous sclerosis complex

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SIR-

Tuberous sclerosis complex (TSC) is an autosomal dominant hamartomatosis with multisystem involvement. There are two gene loci. TSC2, on chromosome 16, has been isolated (The European Chromosome 16 Tuberous Sclerosis Consortium 1993) but TSC1, on chromosome 9q34, has not. Whether the type of mutation and the phenotype are correlated in tuberous sclerosis is subject to debate but the large clinical variation within families does suggest that any correlation will not be a simple one. As in neurofibromatosis type 1, another hamartomatosis, part of the difference in clinical severity between affected family members bas been attributed to somatic mosaicism (Riccardi and Lewis 1988). A de-novo mutation in the gene for neurofibromatosis type I has been demonstrated in the germ-line of the symptom-free father of two patients (Lazaro et al. 1994). To our knowledge, neither somatic nor germ-line mosaicism have been proved in TSC.

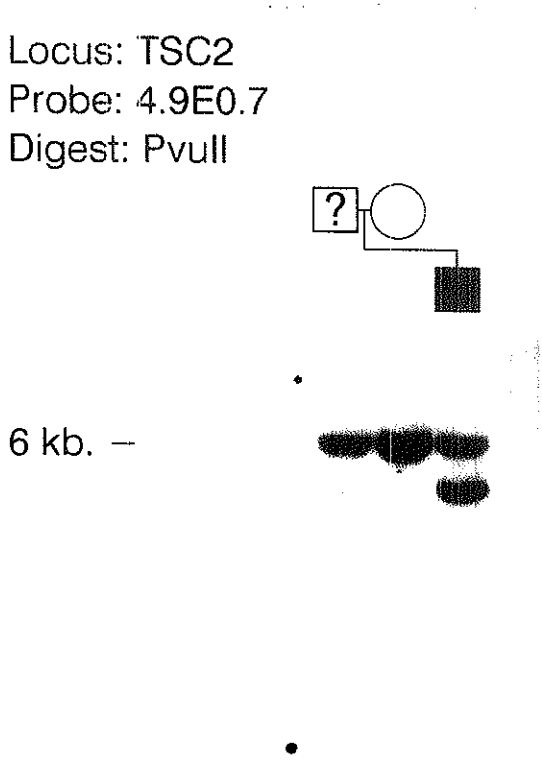
In a TSC nuclear family, a 2-year-old boy was diagnosed as having tuberous sclerosis, with multiple hypomelanotic macules and epilepsy. Computed tomography showed subependymal calcifications close to the foramen of Monro and cortical hypodensities. The father has multiple dental pits and papules in the nasolabial fold, suggestive of adenoma sebaceum. Histological analysis did not prove angiofibroma. On computed tomography one paraventricular calcification was noted, compatible with tuberous sclerosis. He is of normal intelligence, does not have a history of epilepsy, and investigation of heart and eyes showed no abnormalities.

Genomic DNA was isolated from blood samples from the affected child and his parents. Mutation analysis was performed by Southern blotting of genomic DNA digested with restriction enzymes (Sambrook, Fritsch, and Maniatis 1989). The intragenic TSC2 4.9E0.7 probe on PvuII digested DNA deletion of about 1.5 kb in TSC2 was seen as a shortened fragment, with signal intensity equal to that of the normal allele, in the DNA of the affected child. The deletion was confirmed with other restriction enzymes. The abnormal pattern was not present in DNA of the mother. The subclinically affected father shows the same mutation in TSC2 as his affected son, but the aberrant band was much weaker than that for the normal allele (Fig. 3.7). Somatic mosaicism was confirmed in a second blood sample from the father.

In the case of germ-line mosaicism in NF1 (Lazaro et al. 1994) thorough clinical investigation revealed no sign of the disease in the father and mutation was not detectable in peripheral blood leukocytes. In contrast, the father described here has subclinical signs of tuberous sclerosis and an apparently low proportion of blood cells with the mutation.

We propose that in this family somatic mosaicism is a good explanation for the variable clinical expression of TSC between parent and child.

Figure 3.7: Southern blot of TSC2 gene using probe 4.9E0.7 on PvuII digested DNA of the investigated family.



Left lane: mosaic father, middle lane: unaffected mother, right lane: affected son

3.7
High Rate of Mosaicism in Tuberous Sclerosis Complex

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Summary

Six families with mosaicism are identified in a series of 62 unrelated families with a mutation in one of the two Tuberous Sclerosis Complex (TSC) genes, TSC1 and TSC2. In five families, somatic mosaicism was present in a mildly affected parent of an index patient. In one family with clinically unaffected parents gonadal mosaicism was detected after TSC was found in three children. The detection of mosaicism has consequences for genetic counseling of the families involved, as changed risks apply to individuals with mosaicism, both siblings and parents.

Clinical investigation of parents of patients with seemingly sporadic mutations is essential to determine their residual chance of gonadal and/or somatic mosaicism, unless a mosaic pattern is detected in the index patient, proving a de novo event. In our data set, the exclusion of signs of TSC in parents of a patient with TSC reduced the chance of one of the parents to be a (mosaic) mutation carrier from 10% to 2%. In the five families with somatic mosaicism, the parent was given the diagnosis after the diagnosis was made in their child.

Keywords

Tuberous Sclerosis Complex, mosaicism, mutations, somatic, gonadal

Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant hereditary disease, characterized by the growth of hamartomas and hamartias that can occur in almost all tissues. Most lesions occur in the brain (subependymal nodules or giant cell astrocytomas, subcortical and cortical tubers), in the skin (facial angiofibromas, white macules, shagreen patches, or ungual fibromas), in the kidneys (cysts and angiomyolipomas), in the heart (rhabdomyomas) and in the eyes (retinal hamartomas). Patients with severe disease suffer from epilepsy (found in 60%-70% of patients with TSC), and mental retardation, found in 40%-50% of patients with TSC. Mortality due to TSC is mostly from two complications: (1) renal bleeding from an angiomyolipoma or (2) the development of an intracerebral giant cell astrocytoma. The prevalence of tuberous sclerosis complex is estimated at ~1/10,000 births. More than half of patients with TSC represent sporadic cases, reflecting a high mutation frequency, estimated at 2.5/10,000/gene in each generation (Sampson et al. 1989a).

TSC is caused by a mutation in either the TSC1 gene, located on chromosome 9 (MIM191100), or the TSC2 gene, on chromosome 16 (MIM191092). After both genes were characterised, the identification of mutations became feasible. Mutation analysis for both genes reveals that most families have a unique mutation, although recurrent mutations have been described (Au et al. 1998; van Slegtenhorst et al. 1997; van Slegtenhorst et al. 1999; Verhoef et al. 1998a) Mutation detection has provided an opportunity to study possible relationships between genotype and phenotype, to investigate presumed de novo mutations, and, occasionally to provide diagnostic certainty in individuals with a particularly mild clinical phenotype.

Reports of "apparent nonpenetrance" in TSC, with multiple offspring of clinically unaffected parents, suggested that parental mosaicism could be expected, as had been suggested by Hall and Byers (Hall and Byers 1987). Several examples of somatic (Verhoef et al. 1995; Sampson et al. 1997b) and gonadal (Yates et al. 1997) mosaicism in TSC have been reported.

We present six families in which mosaicism was detected in a group of 62 unrelated families with TSC with a known mutation (from our study population of 225 families). All five individuals with somatic mosaicism had mild phenotypic features of TSC; in one family the mosaicism was proven to be gonadal and, possibly, somatic as well. Clinical data on the affected individuals in the families are presented.

Subjects and Methods

The present study was approved by the Medical Ethics Committee of the Academic Hospital Rotterdam Dijkzigt.

Subjects: Clinical data and blood samples from patients with TSC have been collected from 1987. Data from a total of 350 individuals with TSC from 225 unrelated families have been entered in a clinical registry. Mutation analysis is generally performed with the use of DNA

from the index patients of each family. Details of the affected family members presented in this paper are discussed in the Results section.

Methods: Mutation analysis was undertaken in 225 unrelated patients with TSC. The DNA of affected individuals (and parents, when available) was isolated from peripheral leukocytes obtained by venipuncture. Mutation analysis of the TSC1 gene consisted of SSCP analysis of all coding exons. Primer sequences used for the amplification reactions are those described by van Slegtenhorst et al. (1999). Direct-sequence analysis was performed according to standard methods. For the identification of mutations in the TSC2 gene, Southern blotting was used to detect large-size abnormalities, followed by SSCP analysis for all coding exons (still in progress). Relevant primer sequences are given in table 3.9; the other exons primers are used as published elsewhere (Au et al. 1998).

Southern blotting was done according to standard procedures by means of genomic DNA digested with the restriction enzymes HindIII and EcoRI. Probes used for analysis of large deletions in the TSC2 gene are 4B2, 4.9E0.7 and 1A1 (The European Chromosome 16 Tuberous Sclerosis Consortium 1993).

Oligonucleotides were designed for the normal and the mutated sequences (Table 3.9). Allele-specific oligonucleotide (ASO) hybridizations were performed on duplicate filter sets at 37°C for 30 min. Filters were washed to 0.3 x SSC for 10 min at 37°C.

For haplotype analysis in family 2, flanking markers were used on chromosome 9: D9S2126 and D9S1830, proximal to TSC1; D9S1199 and D9S1198 distal to the gene (van Slegtenhorst et al. 1997). Reverse-transcription (RT)-PCR analysis for family 4 was done on total RNA from fibroblast cells of the index patient according to standard procedures. The primers used in the RT-PCR reaction were forward 5'-GCAAAGATTCAGGCTTGAAGG-3' (exon 1) and 5'-CCACATTCCATGCTCAGT-3' (exon 2). Direct-sequence analysis was performed on the isolated aberrant RT-PCR product with the same primers. Families were tested to confirm family structure using the Profiler Plus Kit (Perkin Elmer), which contains nine highly polymorphic markers from nine different chromosomes.

Results

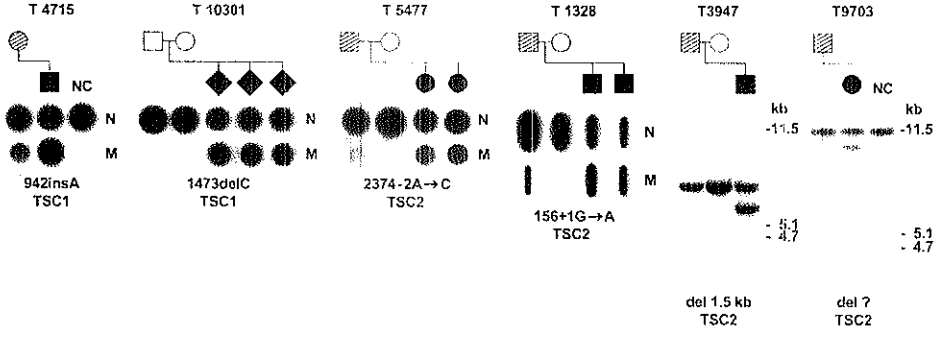
After mutation analysis of the complete coding region of the TSC1 gene, Southern blotting for the TSC2 gene, and SSCP analysis of ~25% of the coding region of the TSC2 gene, 29 mutations were detected in TSC1 and 33 mutations were detected in TSC2. In five of the 62 families parental somatic mosaicism could be proved. In the sixth family, occurrence of TSC in three sibs with clinically unaffected parents was consistent with germ line mosaicism, as no somatic copies could be detected in the parents (Figure 3.8).

Mosaicism for mutations in the TSC1 gene

Family 1 (T4715): A boy had epilepsy, mental retardation, facial angiofibroma, ungual fibroma, white macules, a shagreen patch, and gingival fibromas. Cortical tubers and

subependymal nodules were visible on the CT scan. His mother had normal intelligence and no epilepsy, but, when examined, was found to have facial angiofibroma, white macules and a shagreen patch on the skin. A CT scan of the mother's brain showed subependymal nodules. SSCP analysis showed an abnormal pattern of exon 8 of the TSC1 gene. Direct-sequence analysis resulted in the identification of a frameshift mutation 942insA (van Slegtenhorst et al. 1999). ASO hybridisation analysis showed that the ratio of the mutant-to-normal allele was less in the DNA of the mother than in that of her son.

Figure 3.8: Six Tuberous Sclerosis Complex families showing mosaicism.



From left to right: TSC1 mutations 942insA and 1473delC, and TSC2 mutations 2374-2A→C (dot blot of ASO hybridization analysis),156+1G→A (slot blot of ASO hybridization analysis); a deletion of 1.5kb (probe 4.9E0.7), and a deletion of unknown size (probe 4B2) on Southern blot analysis. In the pedigrees the person with mosaicism is indicated hatched; fully affected non-mosaic individuals are indicated black. NC= negative control sample, squares: males, circles: females, diamonds: sex not indicated for confidentiality.

Family 2 (T10301): The index patient was 9 years old, the sixth child in a large family. His medical history revealed congenital cardiac tumours, detected antenatally with ultrasound after analysis of irregular foetal heart rhythm. The tumours had regressed with age. At age 8 years, myoclonic epilepsy occurred. White macules were then noted on the skin. On the basis of these findings, together with the congenital heart tumours (most probably rhabdomyomas), a clinical diagnosis of TSC was made. A CT scan of the brain showed subependymal nodules and confirmed the diagnosis. The youngest sibling developed epilepsy at age 2 years of age and had three hypopigmented macules on the skin. A CT-scan showed subependymal nodules and an irregular cortical gyration pattern, demonstrative of TSC. Both parents were presumed to be unaffected (by skin examined only; they had no history of epilepsy or learning difficulties). SSCP analysis of the TSC1 gene in the index patient in this family showed an abnormal pattern of exon 12. Direct-sequencing followed by ASO hybridisation analysis showed a frameshift mutation 1473delC (van Slegtenhorst et al. 1999). Subsequent analysis DNA from the complete family with ASO hybridisation analysis identified the mutation in both clinically affected children and in a third sib, aged 10 years, suspected of having TSC

because of developmental delay and possibly the beginning of facial angiofibroma. The mutation could not be detected in DNA from blood cells from the parents, suggesting mosaicism. Using flanking markers to the TSC1 gene, we performed haplotyping of the parents and all children. In addition to the three affected children, two more children were shown to carry the affected allele without the mutation, proving gonadal mosaicism in one of the parents (results not shown). Because the parents do not wish to know their carrier status, the parent of origin cannot be disclosed, and further clinical evaluation of the parents has been postponed.

Mosaicism for mutations in the TSC2 gene

Family 3 (T5477): The elder of two sisters had had epilepsy between the age of 6 months and 1 year. Subsequently, she was diagnosed with moderate mental retardation, facial angiofibroma, white macules, a shagreen patch, and an intracardiac tumour. A CT-scan of the brain confirmed the diagnosis of TSC. Her younger sister had epilepsy with severe mental retardation, facial angiofibroma, and ungual fibromas. She also had a cardiac tumour and renal angiomyolipoma. A CT-scan of the brain confirmed TSC in her as well. Their father appeared to have facial angiofibroma, white macules and a shagreen patch on the skin, depigmentations of the retina, angiomyolipoma of the kidney, and gingival fibroma of the mouth. He had no epilepsy or mental retardation. In this family, SSCP analysis followed by direct-sequencing of intron-exon boundaries led to the detection of a splice-site mutation 2374-2A→C in intron 20 of the TSC2 gene, present at a mosaic level in the father. The effect of this splice-site mutation could not be studied, since no RNA was available.

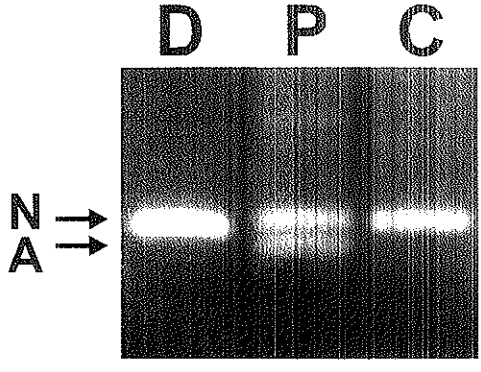
Family 4 (T1328): The oldest son in this family had facial angiofibroma, white macules, subependymal nodules, and cortical tubers. Although he had suffered from infantile spasms, he had normal intelligence. His brother, 14 months younger, had facial angiofibroma, white macules, subependymal nodules, and cortical tubers, with epilepsy and mild mental retardation. He showed cardiac rhabdomyoma and a single gingival fibroma. Facial angiofibroma and white macules were subsequently seen in the father. A CT scan of his brain revealed subependymal nodules. He had no retardation or epilepsy. Mutation analysis resulted in the identification of a splice site mutation 156+1G→A in intron 1 of the TSC2 gene. RT-PCR analysis with primers located in exon 1 and exon 2, resulted in the identification of an abnormal fragment (Figure 3.9). Direct sequence analysis of the aberrant fragment with the same primers showed the use of a cryptic splice site in exon 1 at nucleotide position 105-106 (numbering according to The European Chromosome 16 Tuberous Sclerosis Consortium 1993), causing a frameshift resulting in a stop codon at amino acid position 31. This mutation was previously described in another family (Kumar et al. 1997). The signal of the mutant allele was clearly reduced in the father, shown by ASO hybridisation analysis, indicating somatic mosaicism.

Family 5 (T3947): This family was originally reported as the first TSC family showing somatic mosaicism, proved by DNA-analysis (Verhoef et al. 1995). A 4-year-old boy had

epilepsy for two years and multiple white macules. Periventricular subependymal calcifications and cortical hypodensities were seen on his CT scan, proving the diagnosis of tuberous sclerosis. The father showed mild signs of tuberous sclerosis, with subtle facial angiofibroma, dental pits, and, on CT scan, one paraventricular calcified nodule, but no epilepsy or mental retardation. A 1.5-kb deletion in the TSC2 gene was detected with Southern blot analysis with marker 4.9E0.7 on PvuII digested DNA. A consistent difference in signal intensities between normal and aberrant fragments demonstrated somatic mosaicism in the father. This result was confirmed on a newly collected blood sample.

Family 6 (T9703): The index patient in this family was a 2-year-old girl, given a diagnosis of TSC when analysed for her epilepsy. The parents were then screened. The father was found to have ungual fibromas and facial angiofibroma. A CT scan of the brain revealed several subependymal nodules, and a renal CT scan had showed multiple small angiomyolipomas. The father had no history of epilepsy and had normal intelligence. Southern blot analysis, using probes 4.9E0.7 and 4B2 on DNA of the index patient, indicated a deletion in the TSC2 gene. Despite the use of a combination of different restriction enzymes, the exact length of the deletion could not be determined. Her father has a changed ratio of normal to abnormal signals and carries somatic mosaicism. The clinical data on the individuals carrying the mosaicism are summarised in Table 3.10.

Figure 3.9:



RT-PCR products of a cDNA clone containing the complete coding region of the TSC2 gene (D), from fibroblasts of one of the mosaic tuberous sclerosis complex patient of family 5 (P), and fibroblasts of a negative control (C). N= normal RT-PCR fragment, A= aberrant RT-PCR fragment.

Discussion

In general, a high rate of mosaicism has been predicted for conditions with a high percentage of new mutations (Hall 1988) and has recently been demonstrated for another tumour suppressor gene syndrome neurofibromatosis type 2 (Kluwe and Mautner 1998). Our results

indicate a level of gonadal and/or somatic mosaicism of ~10 percent (6/62) in the group with TSC with a known mutation. The true prevalence of mosaicism in our study population of TSC patients is probably higher than the 10% (6/62) detected in this study because in 17 of the 62 families the parents were not investigated. The proportion of sporadic TSC patients with a severe phenotype who are mosaic is unknown, as most mutations in TSC are unique mutations, and a distinction between high-grade mosaicism and non mosaicism can be impossible. Conversely, a low level of mosaicism in blood cells can be undetectable; thus the mutation will remain unidentified unless other tissues from the patient are tested.

According to empirical, worldwide data on families with TSC, the incidence of gonadal mosaicism in tuberous sclerosis in clinically unaffected parents of children with TSC is ~1%-2% (Berberich and Hall 1979; Baraitser and Patton 1985; Connor, Stephenson, and Hadley 1986; Rott and Fahsold 1991; Webb and Osborne 1991; Ruggieri et al. 1997) In our study, one such family with mosaicism was detected as a result of multiple affected offspring from healthy parents. Gonadal mosaicism was proven by haplotype analysis, whereas the presence or absence of somatic mosaicism could not be investigated extensively. Gonadal mosaicism was recently reported for TSC (Yates et al. 1997). Both in the family reported by Yates and in the family we studied, it remains uncertain as to whether the mosaicism was present in the gonads only or in other tissues as well.

In all five members in whom somatic mosaicism was detected, the diagnosis of TSC was made in their children first. Subsequent clinical investigations of the parents showed that the mosaic parents did fulfil the diagnostic criteria of TSC (Roach et al. 1992), although all these parents had normal intelligence and no epilepsy.

For the individual with mosaicism, the risk for future offspring to inherit the mutation is dependent on the proportion of germ cells that contain the mutation. This risk will be ≤50%, as has been shown for other conditions (Zlotogora 1998). An exact estimate of the level of mosaicism in the gonads is difficult to obtain, although sperm analysis is technically possible. Siblings and parents of a patient with proven mosaicism have a population risk of a TSC mutation, because a mosaic mutation is presumably a post-zygotic event, and thus excludes gonadal mosaicism in one of the parents as a source of the mutation.

Complete clinical screening of parents of seemingly sporadic patients with TSC remains essential in order to exclude the possibility of a mild phenotype of which parents are not aware, to which somatic mosaicism might contribute. If parents do not show any signs of TSC at full clinical evaluation (including a brain CT scan), the chance of one of them having gonadal mosaicism would be ~2% (in our data set 1 family in 45). A substantially higher possible overall mosaicism figure (10% in our data set) might need to be given until parents have completed clinical investigations and have shown no abnormalities.

Since low-degree somatic and gonadal mosaicism can remain undetected in DNA from blood cells, we discuss the low-percentage chance of gonadal mosaicism with parents of a child with TSC seemingly due to a de novo mutation and would be inclined to offer prenatal testing for the mutation in a further pregnancy if requested by the parents. In view of the serious

consequences for genetic counseling of families with TSC, a larger series of mosaic and nonmosaic parents would be required, with a more systematic genotype-phenotype analysis, to show whether somatic mosaicism contributes to a milder phenotype in TSC.

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Electronic Database Information

URL for data in this article is as follows: Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim (for TSC1 [MIM191100] and TSC2 [191092]).

Table 3.9: Description of oligonucleotides for the amplification of exons for SSCP analysis and ASO hybridizations.

Mutation	Exon SSCP primer		Mutation ASO primer	
TSC1	Exon 8		942insA	
	Forward	5'-attcagccctttataatttgtcaac-3'	normal	5'-ggaccatgaactgg-3'
	Reverse	5'-ctcctagatcacattttcaatctctc-3'	mutant	5'-ggaccatgaaactg-3'
TSC1	Exon 12		1473delC	
	Forward	5'-tgttctgcccttgtctctaag-3'	normal	5'-tcacacccccag-3'
	Reverse	5'-agtgagtcactgtgcctgg-3'	mutant	5'-tcacacccccagg-3'
TSC2	Intron 1		156+1G→A	
	Forward	5'-cagaggtgttgctcagatgtccc-3'	normal	5'-atactgagagtgagtga-3'
	Reverse	5'-atttccctctagcctagcaaaga-3'	mutant	5'-aatactgagaatgagtga-3'
TSC2	Intron 20		2374-2A→C	
	Forward	5'-ggcctgaggtgtcctgtct-3'	normal	5'-tcctgcagcgcga-3'
	Reverse	5'-agtgctgcagggcggggac-3'	mutant	5'-tcctgccgcgcga-3'

Table 3.10: Clinical signs of the person mosaic for the mutation (for family 2 both parents are given).

Family lab number	4715	10301		5477	1328	3947	9703
Family number	1	2*		3	4	5	6
Relationship to index	mother	father	Mother	father	father	father	Father
Number of affected children	1	3	3	2	2	1	1
Facial angiofibroma	+	-	-	+	+	+/-	+
White macules	+	-	+/-	+	+	-	-
CT-scan abnormalities	+	u	u	u	+	+/-	+
Intelligence	n	n	n	n	n	n	n
Epilepsy	-	-	-	-	-	-	-

n=normal intelligence, +/- single symptom/minimal sign, u=unknown
* both parents clinically unaffected, but incompletely examined

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CHAPTER 4

DISCUSSION

4.1
General remarks: history, prevalence and differential diagnosis of TSC

At the beginning of the research project on Tuberous Sclerosis Complex, the aim was the identification of the disease causing genes. The results of the gene identification studies were described in separate theses (Janssen 1995; van Slegtenhorst 1998). TSC was chosen as the disease of study, as it could be clinically demarcated from other types of hereditary epilepsy syndromes and occurred both in reasonably large families and in sporadic patients. Large families were necessary for gene localisation studies by linkage analysis, and subsequently mutation analysis of candidate genes required large numbers of independent 'de novo' patients/families. Because of the occurrence of familial and sporadic cases with extensive clinical variability, the clinical and molecular genetic study of TSC might provide model experience for later studies of more common epilepsies and other disorders with complex inheritance. Furthermore, the Dutch paediatric neurologist Dr. P.F. Fleury had already collected a number of TSC families, providing a good basis for genetic studies. As soon as it became clear that more than one gene was involved in TSC, the disease became also a model for other genetic disorders with locus heterogeneity. It was clear that, due to the wide clinical variability between affected family members, studies of the relationship between genotype and phenotype would not be straightforward. Little is yet known about possible modifier genes for TSC, which may play a role in determining which sites will be affected, to which degree and at what age.

Prevalence of TSC

In the available literature, estimates for the birth prevalence of TSC vary between 1/6,000 and 1/10,000. Based on these figures, about 20-30 patients with TSC are born in the Netherlands each year. Assuming close to normal average life expectancy, some 1500-2500 Dutch people would be currently affected with the disease. During the study period between 1988 and 1998 data of 382 patients, including some patients from abroad, with Tuberous Sclerosis Complex was obtained (Chapter 2.2). Thus, the current study is far from complete in its ascertainment of all Dutch cases. The analysis of the clinical data of these patients provided data on the relative prevalence and age of onset of clinical signs and symptoms. It is expected that especially very mildly affected individuals and severely affected institutionalised patients will be underrepresented in the study population. As with most other non-population-based studies, ascertainment bias towards more severely affected individuals is likely. The results of our analyses are largely in line with the already published data and confirm that TSC is a truly complex disease. In our study, 58% (220/382) of the patients with TSC were sporadic cases, presumably due to new mutations in either TSC gene.

Differential diagnosis

Most physicians have very limited practical experience with and knowledge of TSC. Because of its low frequency, on average less than one in every 5 general practitioners will have a TSC patient in his or her practice. Therefore, it seems reasonable to propose that clinicians suspecting TSC in a patient refer to a specialised clinician. Needless to say that any delay of correct diagnosis of TSC may have serious drawbacks for the patient as well as for the relatives, due to delay of preventive follow-up examination and genetic counselling. TSC is a complex disorder and encompasses multiple organ systems; therefore, the medical care for patients with TSC often requires input from many different medical specialities. At the time of making the diagnosis, the differential diagnosis of TSC has to be considered in relation to both the presenting sign(s) and the age at which the index patient is examined (see also Table 1.3). In Chapter 2, we reported two histories of a family and a case referred to us with the diagnosis of TSC, who in fact did not suffer from TSC, but from cylindromatosis and familial periventricular nodular heterotopias respectively. In both cases, the correct diagnosis was made through re-evaluation of all available data in a setting of extensive clinical and clinical genetic experience. In the case of cylindromatosis, not enough attention had been paid to the suggestions in the original pathological report. However, persisting problems manifest in the face or on the scalp, without occurrence of any other sign of TSC among a large number of relatives, in combination with linkage analysis led to the exclusion of TSC. A correction of the diagnosis into cylindromatosis was made and could be proven by the identification of the disease-causing mutation in the CYLD1 gene. In the case of familial periventricular nodular heterotopia, follow-up examinations showing absence of calcification of periventricular nodules and recognition of subtle signs like a history of patent ductus arteriosus led to the correct diagnosis of FNH. Whether the partially overlapping symptoms in TSC, cylindromatosis or familial periventricular nodular heterotopia reflect partly common molecular pathways remains an intriguing question to be answered in the near future.

Although the section concerning the differential diagnosis (Chapter 2), was focussed on patients or families referred with an incorrect diagnosis of TSC, one should equally be aware of the opposite. Sometimes, TSC is mistaken for another disorder, or a presenting sign of TSC is misinterpreted as an entity of its own. We have seen a patient who had suffered from epilepsy and had intracranial calcifications on his brain CT-scan, and was referred with the diagnosis of cerebral toxoplasmosis. Before his referral to the department of clinical genetics, he had the impression that he had a non-hereditary disease with no increased risk for his children. However, when it turned out that he had TSC, he was confronted with a 50% recurrence risk of TSC. In other undiagnosed TSC patients, surgical removal of kidneys with angiomyolipoma has occurred because of wrongly suspected renal malignancy.

4.2
Assessment, DNA-analysis, genetic counselling and prenatal diagnosis of TSC

Recommendations on initial work up and follow-up intervals for both symptomatic and asymptomatic individuals with TSC were formulated by a panel of experts at a consensus conference in 1999 (Roach et al. 1999). The panel recommended that at the time of diagnosis of TSC, full examinations should be done in order to establish a good clinical overview of the initial status of the signs and symptoms of TSC. Repeat testing is advised, depending on the organ system affected, and on the severity of the abnormalities (Table 4.1).
The consensus conference panel concluded that screening only makes sense if the findings have clinical implications, thereby limiting the extent of post-diagnosis monitoring. Obviously, for a proper application of this rather flexible guideline, extensive knowledge of and experience with TSC is asked from the physician co-ordinating the follow-up. The guidelines imply that the recommended follow-up examinations for asymptomatic children with TSC consist of regular renal ultrasound and cranial MRI-scan or CT-scan every 1-3 years only. For the children with TSC who have epilepsy, monitoring of seizure control and complications of the anti-epileptic medication and assessment on mental development are important. In asymptomatic adults, investigations are limited to regular renal ultrasounds. It is illustrated in Chapter 2.4, that regular follow up of the renal lesions into adulthood is indeed worthwhile, in view of the dangers of retroperitoneal bleeding.
In our study group some patients were diagnosed with astrocytoma at adult age, an observation that to our knowledge has not been reported in other studies. Therefore, we would recommend periodic follow up for adult individuals with subependymal nodules located close to the foramen of Monro by repeated CT scan or MRI scan, with an interval of 1-3 years, in order to monitor the possible progression of paraventricular lesion into obstructing giant cell tumour. It has been suggested that early operative treatment, consisting of (stereotactic) brain surgery at the time of the detection of growth of the lesions provides the best treatment (Torres et al. 1998). In our series we registered several cases of late surgical removal of giant cell astroctyoma which were complicated by severe postoperative bleeding, causing neurological deficit or even death. The concentration of early elective neurosurgical treatment of giant cell astrocytoma in centres of expertise may be an important step leading to a reduction of post-operative morbidity and mortality.
Besides monitoring of the disease symptoms from the point of view of 'care' and to improve existing protocols, a better knowledge of the natural course and variation of TSC must be obtained. Over the last years, in a number of countries 'centres of expertise' have been founded incorporating clinics for the follow-up of TSC patients. These outpatient clinics provide a huge benefit, because expertise of TSC in all its diversity is concentrated. One of the great challenges of these centres of expertise for the near future is to establish a system or method to disseminate knowledge about TSC into the medical community at large. Currently,

patients and their parents are often better informed than the average physician and it is not uncommon that they tell their physician about the specialised clinic, TSC-websites, or about the TSC patient organisation, rather that the other way around.

Table 4.1 Recommended investigations in TSC patients (Roach et al., 1999)

Assessment	Initial Testing	Repeat Testing
Neurodevelopment testing	At diagnosis and at school entry	As indicated
Ophthalmic examination	At diagnosis	As indicated
Electroencephalography	If seizures occur	As indicated for seizure management
Electrocardiography	At diagnosis	As indicated
Echocardiography	If cardiac symptoms occur	If cardiac dysfunction occurs
Renal ultrasonography	At diagnosis	Every 1 to 3 years
Chest computed tomography	At adulthood (women only)	If pulmonary dysfunction occurs
Cranial CT scan	At diagnosis	Children/adolescents: every 1-3 years
Cranial MRI	At diagnosis	Children/adolescents: every 1-3 years

Until the present, reliable long-term follow-up data on the natural course and frequency of the different signs and symptoms have been scarce. Some studies have focused on the natural course of a specific (subgroup of) symptom(s), e.g. on the natural course of renal lesions (Ewalt et al. 1998), cardiac rhabdomyomas (Farooki et al. 1991; Jozwiak et al. 1994; Holley et al. 1995), or of symptoms of the eyes (Szreter, Jozwiak, and Michalowicz 1994). The TSC-clinics present an opportunity to gather data on less obvious health hazards for persons affected with TSC, like malignancies or behavioural disorders.

Although the association between hamartomatous syndromes and malignant disease is known, the risk of developing cancer, and whether this chance is variable for different sites, is not clear. Following the identification of both TSC genes, loss of heterozygosity (LOH) in TSC lesions, like angiofibromas, astrocytomas (albeit less frequently) and angiomyolipomas, supported the idea of a tumour suppressor function of the genes. At present, the pathway of tumorigenesis in which the TSC1 and TSC2 genes are involved is unclear. The case history of the patient presented in Chapter 2.3, in whom loss of heterozygosity (LOH) of the 'wild type' TSC2 gene in a rare pancreatic tumour was shown, and the demonstration of a TSC1 mutation in bladder tumours from non-TSC patients (Hornigold et al. 1999), both suggest that the TSC genes are involved in pathways of general importance in tumour development. Similarly, LOH of chromosome 16 (less often chromosome 9) in e.g. pulmonal lymphangioleiomyomatosis (LAM) and the identification of somatic mutations in affected

LAM tissue (Carsillo, Astrinidis, and Henske 2000), have added evidence that outside the context of TSC, the TSC gene products play a role in pathways of tumorigenesis and tumour progression.

DNA testing

DNA analysis is an important tool in the process of genetic counselling. Due to its complexity, mutation screening for the TSC genes can not be offered as a primary diagnostic tool. In new families, clinical investigations should precede DNA-analysis. However, in families with an identified mutation, DNA diagnosis can be used for predictive testing, including prenatal diagnosis, replace extensive clinical work-up protocols for relatives, and provide information on the important issue of possible somatic or germ-line mosaicism.
At this moment, in a fixed research population and using a combination of techniques, a 70-83% mutation yield can be achieved, as has been reported in the literature. A number of the mutations identified in the TSC1 and TSC2 gene from our study population have been described in Chapter 3. In our laboratory, the allele specific oligonucleotide (ASO) hybridisation assay has proven to be a robust and reliable technique for confirmation of point mutations and testing of relatives. ASO hybridisation analysis has the additional advantage that it allows for the detection of reasonably low levels of somatic mosaicism. We observed somatic or germ-line mosaicism in 6 out of 62 identified mutations, suggesting that mosaicism might be a quite frequent phenomenon in new patients or in one of their parents, even when they are asymptomatic (Chapter 3.7). In the future, a more advanced technology, possibly DNA-chips, will provide rapid mutation detection, but clinical diagnostics will remain important. Regarding the question of genotype-phenotype relation between TSC1- and TSC2-related disease, we found no significant phenotypic differences, but a tendency towards a slightly 'milder' spectrum for TSC1, in agreement with recent literature on this subject. However, the biases involved in the still unexplained difference in observed TSC1 and TSC2 mutation ratio between sporadic patients (20% TSC1 versus 80% TSC2) and familial cases (50% each) account for at least some of the reported differences.

Genetic counselling

For genetic counselling of a patient with TSC and his or her relatives, accurate clinical assessment is necessary, although most TSC patients, once familiar with the spectrum of TSC, will recognise their own skin signs, even before clinical investigations are done. Mutation screening for the TSC genes is still labour-intensive and time-consuming. Therefore, a clinical diagnosis should precede DNA-analysis. In families with an identified mutation in the TSC1 or TSC2 gene, DNA-testing can replace clinical screening in asymptomatic relatives and give a definite result, which is especially valuable in the case of very young relatives or young patients with minimal expression of TSC. Often parents and legal representatives of institutionalised patients hope that DNA-analysis can prevent clinical investigations of the patient, and are rather hesitant when asked if the patient (index case) can be accessed for

verification of the diagnosis by full clinical examination. As long as a mutation has not been identified, the most important step is the establishment of a certain clinical diagnosis of TSC in the index patient, implying indeed a full clinical assessment.
Identifying a mutation is especially important when counselling parents of a child with tuberous sclerosis who have short-term further child-wish. The possibility of germ-line or somatic mosaicism should be discussed. For parents with a child with TSC, the absence of clinical signs after complete investigations lowers the chance of having another affected child to 1-2% at most, while it might be up to 5-10% otherwise (see Chapter 3.7).
The next phase is extensive screening of the relative(s) who request genetic counselling avoiding that subtle clinical or subclinical signs suggesting a diagnosis of familial TSC are being missed. Cranial CT-scanning is currently the preferred method of investigation in adult patients or relatives, when looking for signs of TSC of the brain (Roach et al. 1999). Although generally MRI-scan is regarded as more sensitive than CT-scan, the number of unrelated or unspecific abnormalities detected is also substantially higher. During the first year of life, myelinisation of the brain is incomplete. Therefore, cortical tubers may not be visible on an early MRI-scan and for the exclusion of TSC the MRI-scan should be repeated after the age of one year. The consensus panel noted that it is uncommon to detect brain abnormalities in a person who does not show any physical sign at detailed external examination, consisting of skin examination with ultraviolet light and retinal examination with dilated pupils. However, in adult patients the ultraviolet 'Wood' light (wavelength 360nm) has limitations like the MRI-scan, with a comparably high number of false positive or aspecific signs. Renal ultrasound is always advised, because of the medical relevance of detecting large (>35mm) renal angiomyolipomas.
From our own experience and from recommendations in the literature, it seems sensible to investigate children at risk of having TSC, those with clear symptoms of TSC and those with a mutation by regularly for possible signs. In the first year of life: 3-6 monthly investigations of the skin (Wood lamp) and kidneys (ultrasound) is recommended, and one assessment of eyes (peripheral retina), heart (ECG and ultrasound, only to be repeated when abnormalities are present), and central nervous system (ultrasound and/or MRI-scan) are advised. After the first year, the MRI scan should be repeated, and between 1 year and 6 years, 1-3 yearly examination is advised, depending on which symptoms are present. Between 6 and 16 years one initial complete work-up (ECG and cardiac ultrasound investigation only when auscultation is abnormal) is advised, with repeat testing at 16 years. For the adult individual, heart and dental examinations can be considered optional, to be performed only when in doubt about the diagnosis. Dental examination is not included in the basic set of diagnostic work-up, but has been useful in some families when doubt about a diagnosis persisted in a relative with minimal signs.

Prenatal diagnosis

Until recently, prenatal diagnosis for TSC was only possible for exceptional, large families in which linkage analysis was reliable. In total, until mid 2001 in our department, prenatal diagnostic DNA-testing has been performed in 13 pregnancies, from 8 unrelated families (5 sporadic, 3 familial).

For most families, even in the situation of an affected parent and a prior risk of 50% of an affected child, little could be done except await the outcome of the pregnancy and the infant's development during the first years of life. Occasionally unusual foetal movements (due to intrauterine epileptic seizures) were felt during pregnancy and a prenatal diagnosis of TSC was made. Since the second half of the 1980's foetal ultrasound monitoring was introduced, which made prenatal diagnosis of TSC sometimes possible through the detection of heart or brain abnormalities.

In TSC families without an identified mutation and which are too small for linkage analysis, prenatal DNA-diagnosis can not be offered. Therefore, in high-risk pregnancies (i.e. in case of a 50% risk of an affected foetus), the only available option is an attempt at early diagnosis by foetal imaging techniques. Using prenatal ultrasound, TSC can sometimes be detected around the 20th week of pregnancy. The detection of multiple intracardiac tumours is highly indicative for an affected foetus. Ultrasound may also reveal intracerebral pathology later in pregnancy, especially when calcified paraventricular nodules are present. As no harmful effects are known from ultrasound investigations and the medical expertise is relatively widespread, this method can also be offered to women with a previous child with a seemingly 'de novo' mutation and an estimated recurrence risk of a few percent. However, a normal result of ultrasound investigations at 20 or 24 weeks does not exclude TSC or guarantee a favourable outcome of the pregnancy.

Another powerful and accurate diagnostic imaging technique is MRI scanning, especially useful for the detection of brain lesions. The first report of the use of MRI scanning in the antenatal diagnosis of TSC was by Mirlesse et al. (Mirlesse et al. 1992). In most studies, sedation of the mother with flunitrazepam (Rohypnol®) was used in order to ensure a reasonably still lying foetus, thus avoiding disturbing movement artefacts (Revel et al. 1993). By this method, TSC was detected between the 21st and 35th week, consisting of cardiac and/or central nervous system abnormalities. In only one third of the cases the detection was before a pregnancy duration of 24 weeks (Sonigo et al. 1998), after which termination of the pregnancy on medical grounds is not allowed under Dutch law.

When women undergo late foetal ultrasound investigations for reasons unrelated to TSC, occasionally intracardiac tumours are detected. Rhabdomyomas are estimated to make up 50%-60% of the multiple intracardiac tumours detected during routine ultrasound or after analysis for irregular foetal or neonatal heart rhythm (Chitayat et al. 1988; Holley et al. 1995; Abushaban, Denham, and Duff 1993; Webb et al. 1994; Sallee et al. 1999). In the group of prenatal or congenital cardiac rhabdomyomas (without a family history of TSC), the proportion of TSC patients was estimated between 51% and 86 % (Harding and Pagon 1990).

Thus, infants with TSC due to a 'de novo' mutation are sometimes identified before birth, although confirmation by postnatal clinical evaluation is needed. This confirmation may take several months, and is obviously a very tense period for the parents, who have to wait whether white macules appear, cranial imaging reveals abnormalities, or infantile seizures occur. Sometimes, the diagnosis TSC in the foetus may be indirectly made or corroborated by typical TSC signs or symptoms in one of the parents, who was not aware of their presence or meaning.

After the identification of both TSC genes and the subsequent availability of mutation detection, the number of families in whom reliable prenatal DNA-diagnosis could be offered has increased. However, in (autosomal) dominant diseases with wide intrafamilial variation in phenotype, like TSC and neurofibromatosis type 1, affected parents often find it very difficult to decide about a pregnancy of an affected foetus. Therefore, prenatal diagnosis has not been asked frequently . Sometimes, couples ask for testing in order to prepare, and want to take the risk associated with invasive prenatal diagnosis, and such requests should be considered against the background of a risk of miscarriage and because it means predictive testing of a child before it can decide for itself.

The advances of DNA technology provide early and reliable diagnosis on foetal tissue, taken by chorionic villus sampling in the 11th-12th week. In the majority of cases, a couple requesting prenatal diagnosis can have the result in time for termination of the pregnancy by curettage. When an affected pregnancy is continued, monitoring is done using ultrasound. For parents with a child due to a seemingly 'de novo' mutation, there may be still an up to 10% increased risk for another affected child because of the possibility of germ line or somatic mosaicism in one of the parents (see Chapter 3.7 and the previous passages), so invasive prenatal diagnosis is offered.

Prenatal DNA-analysis can also be offered in families with an unknown mutation, but large enough to enable reliable linkage analysis. Prior to prenatal diagnosis, testing of both partners for informativeness for the DNA microsatellite markers is necessary. It is important to ensure that parents understand the possible limitations of the method. On one occasion a complicated situation occurred, when prenatal diagnoses by linkage analysis of chromosome 16 was performed in a large family. A complex uniparental disomy of the normal and risk chromosome 16 of the affected mother was present, but the TSC2 region clearly showed 2 copies of the normal maternal chromosome (Los et al. 1998). The situation was explained to the parents. After a tense pregnancy and delivery, a healthy albeit small child was born.

4.3
Suggestions for further studies

The current study clearly demonstrates the great difficulties that can be encountered when aiming for nation-wide studies. By describing the successive aims of the study, the influence they had on the kind of patients included (also affecting the quality of the available clinical information), various problems can be identified. Important limiting factors when trying to gain full knowledge of TSC are:

a) A lack of reliable national health care registries including inpatient as well as outpatient diagnoses
b) The low level of experience of doctors with the clinical recognition of 'milder' TSC
c) The incomplete knowledge of genotype-phenotype relations of TSC1- and TSC2 related TSC
d) The incomplete resolution of the current TSC1 and TSC2 mutation analysis
e) The lack of sufficient knowledge of the (molecular) pathogenetic mechanisms and pathways underlying in the different manifestations of TSC
f) The unknown effects of mutations in genes encoding products that interact with the TSC gene products .

In order to move forward and increase our knowledge about the clinical aspects of TSC, data should be pooled and well defined research populations of TSC patients should be established, both mildly and severely affected patients. The international consensus guidelines of 1999 and 2000 can serve as a basis for diagnostic criteria and standardised data collection. For a more detailed study into the natural course of TSC however, these protocols may need extension. In order to set up an epidemiological study of the prevalence of symptoms in TSC, and to be able to construct proper age of onset curves, a group of (initially young) patients should be closely followed up with fixed intervals of assessment, in a special clinic or under central guidance of such a clinic. Sporadic patients will be diagnosed only after the manifestation of one or more clinical problems like epilepsy, developmental delay or skin problems. Therefore the early development of subtle signs can only be studied in children from known TSC families. In families with a known mutation, gene mutation carriers can be followed up from birth (or even before birth), in order to determine the natural course of TSC at young age (Table 4.2). Close monitoring in the first months of life is important, especially for early signs of infantile spasms, which often start with subtle absences (staring gaze). Immediate treatment of infantile spasms should be attempted, as there are indications this increases the chance of successful seizure control, and potentially reduces the risk or the severity of mental retardation. The heart should be monitored right from foetal development into the first years of life, until full regression has occurred or no more changes are seen. Special attention for change is needed around the life periods of hormonal changes, since

there is some anecdotal evidence that cardiac rhabdomyomas may recur. Skin examination is advocated using a Wood lamp with intervals of 3 to 6 months in the first year, yearly until age 10 years, and 2-5 yearly beyond 10 years. Eye examination can be performed at birth or at diagnosis only, and followed-up when lesions are detected at age 4 and 18 years. Imaging of the brain should be done at birth or at diagnosis, repeated at age 1 year (MRI-scan), and when abnormalities are seen with 2 yearly intervals until age 18 and at adult age every 5 years, unless large subependymal nodules that need monitoring are present. Renal ultrasound is recommended at birth, every 6 months in the first year, yearly until age 18 and afterwards in case of large angiomyolipomas, 2-yearly otherwise. Monitoring of the epilepsy by EEG is to be considered every 3-6 months when seizures occur or are suspected, and may be discontinued after normal EEG's for two years. Neurodevelopment testing is proposed at age 4 (school entry) and at entry of secondary school, if not normally done through the school system. Dental examination can be delayed until the appearance of the secondary teeth, and in case of abnormalities followed up 5-yearly. Chest CT-scan should be done once in males, and 5 yearly in female patients before age 50 years.

Table 4.2: Recommended work-up and follow-up schedule for TSC registries

	Birth - 4yrs	4-10 yrs	10-18 yrs	>18 yrs
Heart	3m-6m	1yr	2-5yrs	5yrs
Eyes	once	once	once	once
Skin	3m-6m	1yr	2-5yr	
CT-MRI brain	1yr	2yr	2yr	2-5yr
Kidneys	3m-1yr	1-2yr	1-2yr	2yr
Development		once	once	once
Lungs	-	-	-	once(♂) 5yr(♀)
Teeth	-	-	once	once

Once: at the time of diagnosis only, m = monthly, yr = yearly

More evidence-based programs for preventive examination protocols can be made when these data become available. The costs of these studies may well be paid back through preventive gains of resulting cost-effective protocols, and will provide valuable information for the counselling of patients and parents. In addition, these studies may be a valuable source for studies of the molecular biological mechanisms involved in the clinical expression of TSC.

The molecular pathways in which the TSC1 and TSC2 gene products hamartin and tuberin are involved are a fascinating line of investigation. The discovery of a protein complex including hamartin, tuberin and presumably one or more other partners may provide an important link

with the clinical variability of TSC. Since the clinical variation may be extensive between sibs, the TSC1 or TSC2 gene mutation is apparently not the only (genetic) determinant of the phenotype. Variant other genes, which are commonly called "modifying genes", possibly encoding other components of the hamartin-tuberin complex, may contribute to the phenotypic expression. Genetic and clinical studies aimed at the identification of such modifiers will be complex. Affected sib-pair analysis has been the method of choice in the search for modifiers of the cystic fibrosis phenotype (L.C. Tsui; The European twin and sibling study). Cystic fibrosis as autosomal recessive disease, is an attractive model, in view of the high frequency of patients homozygous for the same mutation in the CFTR gene, whereas several phenotypic parameters show variability. For TSC and other autosomal dominant disorders with a paucity of recurrent mutations, this kind of approach would require the compliance and co-operation of patients, clinicians and geneticists world wide. This may seem a very difficult enterprise, but the mapping and subsequent identification of both TSC genes equally depended on international collaboration. Moreover, it may become increasingly evident that such data are needed, before a start can be made with the design of effective therapies for the treatment of the major complications of this fascinating, but often terrible disease.

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Summary

In this thesis, the results have been presented of molecular and clinical research into the hereditary disease Tuberous Sclerosis Complex (TSC). TSC is an autosomal dominant disease with truly variable expression. The prevalence at birth of TSC is estimated at around 1/10,000. The disease is characterised by the growth of hamartomas: tumours from embryological origin, growing in a disorganised fashion. These hamartomas are usually benign and do not metastasise or show invasive growth into neighbouring tissues, although they often tend to grow slowly and may recur after excision. TSC is one of the neuro-cutaneous disorders, also including the neurofibromatoses type 1 and 2, Von Hippel Lindau's disease and Peutz Jeghers syndrome.

In TSC, the brain and the skin are most often affected. The brain lesions are best visualized with a CT- or MRI-scan. When located in the cortex of the brain as cortical tubers, the hamartomas can be a focus of epilepsy. Subependymal nodules may progress into giant cell astrocytomas, which when located close to the foramen of Monro may cause obstruction of the third ventricle and lead to hydrocephalus. In the skin several manifestations of TSC may be seen. At birth, fibrous forehead plaque and hypomelanotic macules may be present, although white spots may also become apparent in the first year. At slightly later age facial angiofibromas and shagreen patch (often located in the lumbar region) may become evident, and multiple subungual or periungual fibromas of the nails are a specific sign of TSC. Also at birth or even before birth, cardiac rhabdomyoma may be present, sometimes causing heart rhythm disturbance or haemodynamic compromise, which may be life threatening. Intra-cardiac tumours may be seen at ultrasound investigation during pregnancy and as such present the earliest detectable clinical sign of TSC. In TSC, hamartomas have been encountered in all tissue types, except skeletal (striated) muscle. Often pigment disturbances affect the eyes, epecially the peripheral retina, in the kidneys angiomyolipomas and multiple cysts can be seen, and in the mouth gingiva fibromas and multiple enamel pits occur. About one third of patients with TSC belong to a family with other affected individuals. In the remainder two thirds of cases, the patient is the first affected person in his or her family, considered due to a 'new mutation'. Criteria for making the clinical diagnosis of TSC were originally formulated by Gomez, and the modified diagnostic criteria were used for the inclusion of patients in the research project presented in this thesis. Depending on the age at onset and the types of symptoms, confusion with other diseases may occur.

In patients with TSC, one of two genes, TSC1 on chromosome 9 (coding for the protein hamartin), or TSC2 on chromosome 16 (coding for tuberin), is mutated. Mutation analysis for both genes is laborious and consists of a combination of different methods. Most patients have a unique mutation, although recurrent mutations have been reported. Because of the complexity of both the genetics and the clinical diversity in symptoms, genetic counselling of patients and their relatives should preferably be provided through centers with a special interest in TSC. For the screening of (healthy) relatives, extensive clinical assessment is

recommended, unless the causing mutation is known in the family, in which case a DNA test can replace clinical work-up. Prenatal DNA-diagnosis can be offered in those families, and structural ultrasound with the aim of detecting intracardiac tumours is recommended in all high-risk pregnancies. An important pitfall in the genetic counselling process is the fact that a small proportion, (possibly 5%-10% of the patients), is somatic mosaic or has a parent with germ-line mosaicism.

In Chapter 2, clinical data from 370 patients have been presented. The set of data is descriptive rather than suitable for statistical analysis. We were able to confirm that about half of the individuals with TSC have mental retardation and in about 70% of patients epilepsy occurs at some time during their life. Severe mental retardation due to TSC is not observed in non-epileptic patients, unless due to other causes. The diagnosis TSC was known before the age of five years in only a third of the registered patients. The data on mortality show, that early in life cardiac rhabdomyomas, while in the group over 40 years, vascular rupture of blood vessels in hamartomas (spontaneously or post-operatively in angiomyolipoma or following brain surgery for giant cell astrocytoma) are the most frequent causes of death.
During the course of the research project on TSC, a number of unusual manifestations of TSC were presented to us, an example of which is presented in chapter 2.3. In a boy with severe TSC a mixed type neuro-endocrine pancreatic tumour developed. Loss of heterozygosity of the wild type TSC2 gene was demonstrated in the tumour of the patient, strongly relating the tumour to the TSC spectrum of abnormalities in the patient, in whom the primary germ-line mutation in TSC2 gene was identified. In chapter 2.4, the data of a follow-up study into renal manifestations of TSC are presented. The results of this study indicate that preventive treatment (e.g. by embolisation) of angiomyolipomas larger than 3,5 cm is advisory. In the large family described in chapter 2.5, a suspected diagnosis of TSC could be excluded, and a significant contribution could be made to the proper diagnosis of cylindromatosis in this family.

After the identification of the two TSC genes TSC1 and TSC2, both in which our laboratory played a major role as part of a consortium, mutation analysis became feasible. In Chapter 3, the results of mutation studies are presented. Comprehensive screening of the TSC1 gene yielded mutations in 13% of the patients (chapter 3.2). We were able to report the first use of mutation analysis to prove familial TSC in a suspected family, in which on the basis of the clinical criteria alone, the diagnosis could not be made with certainty (chapter 3.3). We reported the first recurrent mutation in the TSC2 gene (chapter 3.5), and the first case of somatic mosaicism, in a mildly affected father of a fully affected child (chapter 3.6). The combination of methods used for mutation analysis in our laboratory allowed us to demonstrate that mosaicism is not a rare event in TSC (chapter 3.7).

In the discussion in Chapter 4, recommendations are made for follow-up of patients with TSC, both from the point of view of medical care and for the purpose of scientific analysis.
Our data show that a correct genetic diagnosis is of the utmost importance and that the possibility of mosaicism may be a complicating factor. Recommendations are made for more detailed and structured clinical studies for assessing genotype-phenotype relations, and for the study of ages of onset of the different signs and symptoms of TSC. It is hoped for the future that knowledge of the metabolic pathways, in which the TSC proteins tuberin and hamartin are involved, may lead to clues for the treatment of at least some of the complications of this serious and complex disease.

Samenvatting

In dit proefschrift worden de resultaten weergegeven van moleculair en klinisch onderzoek naar de erfelijke ziekte Tubereuze Sclerosis Complex (TSC). TSC is een autosomal dominante aandoening met een buitengewoon variabele expressie. De geschatte prevalentie bij de geboorte van TSC is ongeveer 1/10.000. De ziekte wordt gekenmerkt door de groei van hamartomen: tumoren van oorspronkelijk embryonale origine, met ongeorganiseerd groei patroon. Deze hamartomen zijn normaliter goedaardig, zaaien niet en groeien door in het omliggende weefsel. Wel neigen ze vaak wel tot langzame groei en kunnen terugkeren na operatieve verwijdering. TSC behoort tot de neuro-cutane aandoeningen, een groep van hamartomatosen waartoe ook de neurofibromatosen type 1 en 2, de ziekte van Von Hippel Lindau en Peutz Jeghers syndroom behoren.
De hersenen en de huid zijn het meest frequent aangedaan bij TSC. De hersenafwijkingen worden gezien op een CT scan of MRI scan. Bij lokalisatie in de hersenschors als zogenoemde corticale tubers kunnen de hamartomen fungeren als epilepsiebron. Subependymale noduli kunnen overgaan in reuscel-astrocytoom, dat wanneer het in de buurt ligt van het foramen van Monro de derde ventrikel kan blokkeren en zo kan leiden tot hydrocephalus. Aan de huid kunnen verschillende verschijnselen van TSC voorkomen. Bij de geboorte kunnen fibreuze plaques en hypomelanotische vlekken aanwezig zijn, ofschoon de witte vlekken ook vaak in het eerste levensjaar pas duidelijk worden. Op wat hogere leeftijd kunnen faciaal angiofibroma en peau de chagrin (vaak in de lumbale regio) zichtbaar worden, terwijl multipele subunguale of periunguale fibromen van de nagel(riem) een specifiek teken zijn van TSC. Bij de geboorte of zelfs voor de geboorte, kunnen in het hart cardiale rhabdomyomen worden gezien, die hartritmestoornissen en haemodynamische consequenties kunnen geven die soms levensbedreigend zijn. Intracardiale tumoren kunnen in de zwangerschap met echografisch onderzoek worden ontdekt, en zijn derhalve het vroegst detecteerbare klinische teken van TSC. Bij TSC kunnen hamartomen voorkomen in alle weefseltypen, met als uitzondering het dwarsgestreepte skeletspierweefsel. In de ogen worden vaak pigmentstoornissen gezien, vooral van de perifere retina; in de nieren kunnen angiomyolipomen en multipele cysten voorkomen en in de mondholte putjes van het tandglazuur en fibromen van de tandlijsten. Ongeveer een derde van de patiënten met TSC komt uit een familie met meerdere aangedane personen. In de resterende tweederde van de situaties is de patiënte de eerst aangedane persoon in de familie, waarschijnlijk veroorzaakt door een nieuwe of '*de novo*' mutatie. Criteria voor de diagnose TSC werden oorspronkelijk geformuleerd door Gomez. Voor de inclusie in het onderzoek van de patiënten die in dit proefschrift wordt gepresenteerd, werden in latere jaren aangepaste criteria gebruikt. Afhankelijk van de leeftijd en het type symptomen kan verwarring met andere ziekten optreden.
Eén van twee genen, TSC1 op chromosoom 9, band 9q34.3 of TSC2 op chromosoom 16, band 16p13.3, is gemuteerd bij patiënten met TSC. Mutatie analyse van de beide genen is

arbeidsintensief en bestaat uit de toepassing van een aantal methoden. De meeste patiënten hebben een unieke mutatie, ofschoon bepaalde mutaties meerdere keren voorkomen. Vanwege de complexiteit van zowel de genetische als de klinische aspecten van TSC, kan genetic counseling het best plaats vinden in centra met een bijzondere belangstelling voor TSC. Voor de screening van (gezonde) familieleden wordt uitgebreide klinische beoordeling aanbevolen, tenzij de oorzakelijke mutatie bekend is in de familie, in welk geval een DNA-test klinisch onderzoek kan vervangen. In deze families is prenataal DNA-onderzoek mogelijk, terwijl in alle hoogrisico zwangerschappen prenataal echografisch onderzoek gericht op de detectie van intracardiale tumoren wordt geadviseerd. Een belangrijke potentiële valstrik bij de genetic counseling is dat een klein gedeelte (mogelijk 5-10% van de patiënten) somatisch mozaïek is of een ouder heeft met gonadaal mozaicisme.

In hoofdstuk 2 worden de klinische data van 370 patiënten met TSC gepresenteerd. De verzamelde data zijn meer beschrijvend dan geschikt voor statistische analyse. Bevestigd werd dat ongeveer de helft van de patiënten een ontwikkelingsachterstand heeft, en dat bij tenminste 70% epilepsie optreedt in enige fase van het leven. Ernstige mentale retardatie wordt niet gezien bij niet-epileptische patiënten, tenzij er een andere verklaring voor is. De diagnose TSC was bekend voor het vijfde levensjaar bij ongeveer een derde van de patiënten. De data over overlijden geven aan, dat op jonge leeftijd rhabdomyomen van het hart, en op leeftijd boven 40 jaar bloedingen, in hamartomen (spontaan of na operatie in angiomyolipomen, of na hersenchirurgie voor reuscel-astrocytoom) de meest voorkomende doodsoorzaak zijn.
Tijdens het wetenschappelijk onderzoek naar TSC kwamen een aantal ongebruikelijke manifestaties van TSC onder de aandacht, waarvan in hoofdstuk 2.3 een voorbeeld gepresenteerd is. Een jongen met ernstige TSC ontwikkelde een gemengd type neuronendocriene tumor van de pancreas. Verlies van heterozygotie van het 'wild' type TSC2 gen werd in de tumor van de patiënt aangetoond, in sterke mate een verband aangevend met het TSC spectrum van afwijkingen bij deze patiënt, in wie de primaire kiemlijn mutatie in het TSC2 gen werd gevonden. In hoofdstuk 2.4 worden de data van een follow-up onderzoek naar renale afwijkingen bij TSC gepresenteerd. De resultaten geven aan dat preventieve behandeling (bijv. met embolisatie) van angiomyolipomen groter dan 3,5 cm geadviseerd moet worden. In de grote familie die in hoofdstuk 2.5 wordt beschreven werd de waarschijnlijkheidsdiagnose TSC uitgesloten, en een belangrijke bijdrage geleverd aan de correcte diagnose cylindromatosis in deze familie.

Na de identificatie van de beide TSC genen TSC1 en TSC2, waarbij een belangrijke rol was weggelegd voor ons laboratorium in consortium verband, werd mutatie analyse mogelijk. In hoofdstuk 3 worden de resultaten van mutatie studies behandeld. Volledige screening van het TSC1 gen leverde mutaties op bij 13% van de onderzochte patiënten (hoofdstuk 3.2). De eerste toepassing van mutatieanalyse voor het bewijzen van familiare TSC, in een verdachte

familie waarin op grond van de klinische criteria alleen de diagnose niet zeker gesteld kon worden , is beschreven in hoofdstuk 3.3. De eerste herhaalde mutatie in het TSC2 gen (hoofdstuk 3.5) en de eerste beschrijving van somatisch mozaicisme bij een mild aangedane vader met een volledig aangedaan kind (hoofdstuk 3.6) werden gepubliceerd. Mede door gebruik van de allel specifieke oligonucleotide (ASO) analyse ter bevestiging van mutaties en onderzoek bij familieleden werd duidelijk dat mozaicisme niet zeldzaam is bij TSC, en mogelijk bij 10% van de patiënten met een vermoede nieuwe mutatie voorkomt (hoofdstuk 3.7).

In de discussie (hoofdstuk 4) worden aanbevelingen gedaan voor follow-up van patiënten met TSC, zowel vanuit het perspectief van medische zorg, als ten behoeve van wetenschappelijk onderzoek.
Onze data geven aan dat een correcte genetische diagnose van het uiterste belang is en dat de mogelijkheid van mozaicisme een complicerende factor kan zijn. Aanbevelingen voor een meer gedetailleerde en gestructureerde klinische studie worden gegeven, ten behoeve van een nauwkeuriger analyse van genotype-phenotype relaties, en vastleggen van de leeftijden van ontstaan van de verschijnselen van TSC. Inzicht in de metabole processen waarin de TSC eiwitten tuberine en hamartine zijn betrokken zullen hopelijk in de toekomst aangrijpingspunten geven voor de behandeling van tenminste een aantal van de complicaties van deze ernstige en complexe aandoening.

Dankwoord

Bij de voltooiing van een proefschrift passen gemengde gevoelens, vooral wanneer het alles bij elkaar bijna 10 jaar in beslag genomen heeft. Niet dat er in die tijd niets anders gebeurd is (misschien juist wel teveel). Aan één kant ben ik heel blij dat het klaar is, maar aan de andere kant is de promotie een afsluiting van een periode die de eeuwige student in mij het gevoel geeft een beetje te sterven. Maar goed, het mag ook geen levenswerk worden, en dat gevaar leek op een bepaald moment wel op de loer te liggen. Nu kan ik mij op nieuwe horizons gaan richten.

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Bart en Marjon hebben dermate goed werk geleverd voor hun eigen proefschriften, dat mijn taken daaruit eigenlijk als vanzelfsprekend volgden, en Mark konden wij gelukkig inlijven in onze onderzoeksgroep. Mark, ook het hockeyen was altijd erg gezellig. De analisten Arjenne en Lida hebben het meeste werk aan mij gehad, toen ik die proeven leerde die je zelfs een dokter kan leren. De sfeer op het DNA-lab was altijd erg collegiaal, en ik voelde me er thuis.
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Curriculum vitae

De schrijver van dit proefschrift (Senno Verhoef) werd geboren op 5 september 1963 in 's Gravenhage. Zijn middelbare school opleiding werd in 1982 afgerond met het International Baccalaureate examen na een 2-jarige scholing aan het United World College of the Atlantic (Wales). Daarna volgden:

1982 - 1983	Propedeutisch jaar biologie rijksuniversiteit leiden
1983 - 1990	Studie medicijnen, afgerond met het artsexamen aan de Erasmus Universiteit Rotterdam/ Academisch Ziekenhuis Rotterdam Dijkzigt
1990 - 1991	Militaire dienst, Koninklijke Luchtmacht (Geneeskundige Dienst)
1991 - 1993	Aanstelling project tubereuze sclerosis/ genetic counseling (agnio) 50/50. afdeling Klinische Genetica, Erasmus Universiteit Rotterdam
1993 - 1998	opleiding (agio) tot klinisch geneticus (opleider Prof. dr. M.F. Niermeijer)
1993 - 1999	deelaanstelling voor TSC research ten laste van het Praeventiefonds (later Zorg Onderzoek Nederland) nummer 00-28-17231.
1996 - 1997	buitenlands jaar als Senior Registrar, Department of Medical Genetics van het University Hospital of Wales, Cardiff (Prof.dr. P.S. Harper)
1998 - heden	aanstelling als klinisch geneticus bij het Nederlands Kanker Instituut / Antoni van Leeuwenhoek Ziekenhuis, Amsterdam, met deelaanstelling op de afdeling Klinische Genetica van het Academisch Medisch Centrum Amsterdam (Prof. dr. N.J. Leschot)

De auteur is getrouwd met Yvonne Baerveldt. Zij hebben drie kinderen Eveline, Caroline en Rogier

List of Publications

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