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Tuberous Sclerosis: Between Genetic and Physical Analysis

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Abstract. Tuberous sclerosis complex (TSC) is an autosomal dominant multisystem disorder with extensive clinical variability. Present estimates of the prevalence of TSC suggest that it may exceed 1:6,000. New mutations are frequent, as about 2/3 of all cases are apparently sporadic. Locus heterogeneity has been established, with one gene on chromosome 9q34 (TSC1) and the other on chromosome 16p13.3 (TSC2). The majority of TSC2 mutations are propably subtle alterations. In some cases, somatic and germline mosaicism might be explanations for intrafamilial phenotypic variation and apparent non penetrance. A role of the predicted protein product tuberin in growth suppression would be in agreement with allelic losses observed in tumors of TSC patients. Studies on tuberin using antibodies raised against various parts of the protein can be expected to provide insight into its normal and impaired function.

Key words: Tuberous Sclerosis Complex (TSC), TSC2 gene

CLINICAL ASPECTS

Tuberous sclerosis complex (TSC) is inherited as an autosomal dominant trait and is characterized by the growth of benign tumors (hamartomas), which may affect many organs. The most comprehensive description of the disease has been given by Gomez [1]. The brain, skin, heart and kidneys are often involved. Important clinical features are epilepsy and mental retardation. The vast majority of patients have a history of seizures, which is accompanied by mental retardation in about 50% of the cases [1, 3]. Characteristic lesions are cortical tubers, subependymal nodules and giant cell astrocytomas. Renal complications have been reported in a large proportion of the patients, as 40–80% show (often bilateral) renal cysts and angiomyolipomas [1, 4]. Cardiac rhabdomyomas in particular may be life threatening in children, whereas they may regress later on in life

Table 1 - Criteria for the diagnosis of TSC [from ref. 7 based on ref. 6]

Primary category: definitive (pathognomonic) criteria	
Skin:	facial angiofibromas (adenoma sebaceum) - multiple ungual fibromas
CNS:	cortical tubers[a] - subependymal nodules[b] - giant cell astrocytoma[a]
Retina:	multiple retinal astrocytoma
Secondary criteria	
Skin:	shagreen patches - fibrous forehead plaque
Lungs:	lymphangiomyomatosis[a]
Kidneys:	angiomyolipoma[a-c] - cysts[a]
Heart:	rhabdomyomas[a-c]
CNS:	cerebral tubers[b] - non calcified subependymal nodules[b]
Retina:	single hamartoma
A certain diagnosis of TSC in a first-degree relative[d]	
Tertiary criteria	
Skin:	hypomelanotic macules - confetti-like spots
Kidneys:	cysts[b,c]
CNS:	heterotopic white matter[b] - infantile spasms
Gingiva:	fibromas
Teeth:	enamel pits
Rectum:	polyps[a]
Bones:	cysts[b]
Lungs:	lymphangiomyomatosis[b]
Hamartomas in other organs[a]	

CNS = Central nervous system.

[a] Histologic finding.

[b] Radiologic finding.

[c] Ultrasound.

[d] The affection status of relatives is not used as a diagnostic criterium in our linkage studies.

[5]. Skin manifestations include facial angiofibromas, multiple ungual fibromas, shagreen patches and hypomelanotic macules, and are often of significant diagnostic importance. Criteria for the diagnosis of TSC have been established [6] and are listed in Table 1. The presence of one primary criterion, two secondary criteria, or one secondary and two tertiary criteria confirms the diagnosis. The expression of the disease is highly variable, even within families. Since mild cases may escape identification, authors of prevalence studies considered that their figures are presumably underestimates of the true prevalence. Osborne et al. [3] estimated that the prevalence may exceed 1/6,000. About 2/3 of all cases occur de novo, reflecting a high mutation rate ($2.5\ 10^{-5}$) [7], which is of the same order of magnitude as the mutation rate in, for instance, Duchenne muscular dystrophy and neurofibromatosis I (NFI).

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Genetics and linkage

In the absence of knowledge on the primary defect, TSC has been an obvious target for positional cloning strategies, recently reviewed by Janssen [8]. Linkage studies identified chromosome 9q34 as a candidate region for TSC in 1987 [9, 10] by demonstrating cosegregation of the disease with the ABO blood group locus and the Abelson oncogene. This location was subsequently disputed, as other groups were unable to reproduce these results in their own family material [11, 12]. Gradually, it was suspected that two categories of families may be distinguished: those with linkage to chromosome 9q34 and those with an unrelated locus. Janssen et al. [13] designed a method, which combined the analysis of two separate chromosomal regions and which was designated the "imaginary chromosome approach" (ICA). This and other sophisticated linkage strategies provided evidence for locus heterogeneity [14-16] and several candidate regions for the second locus were postulated. Linkage data implicating chromosomes 11 and 12 [13, 14, 17] turned out to be false positive at a later stage. A large collaborative study involving 128 families contributed by eight TSC research groups confirmed linkage to chromosome 9q34 (TSC1) in about 50% of the families, whereas the remaining 50% were unlinked [18]. A second TSC locus was postulated in the absence of evidence for further heterogeneity [18].

A major breakthrough was accomplished in 1992 when Kandt et al. [19] investigated large non-chromosome-9-linked TSC families and established linkage to chromosome 16p13.3. Interestingly, TSC appeared to be linked to a marker that also showed tight linkage to another inherited disorder; autosomal dominant polycystic kidney disease (ADPKD).

Janssen et al. [20] applied the ICA to evaluate a small set of families (n = 14) selected on the basis of their anticipated information content effective number of informative meioses (EFNIM). Evidence was found for TSC1 (chromosome 9q34) in 65% of the families and for TSC2 (chromosome 16p13.3) in the remaining families without any indication for a third locus [20]. Combining the data from several groups it appears that "TSC1" and "TSC2" are equally represented among familial cases of TSC (communicated at the National Tuberous Sclerosis Association (USA) 20th Anniversary International Symposium, October 1994).

The candidate region for TSC1 was refined to a 5-cM area around D9S64 in the data set of Janssen et al. [20]. The combined data from recent linkage reports are consistent with a location between D9S149 and D9S114 [21] (Fig 1). Individual recombination events showed conflicting evidence, as some of them pointed to a position proximal of ABO and DBH, whereas other data supported a location distal to these markers [21]. An alternative mapping strategy is based on the assumption that the TSC genes act as growth suppressor genes, which may be accompanied by loss of wild-type alleles in tumors. Allelic losses in tumors from TSC patients have been observed, but they occurred within the present consensus region [22, 23]. In future, this approach may contribute to a solution of the controversy concerning the validity of the observed recombination events. Recent efforts include the construction of cosmid contigs [24, 25], the search for mutations in candidate genes [26] and exon-trapping experiments [27] and have been aimed both at the proximal and distal parts of the consensus region. So far, the TSC1 gene has escaped identification.

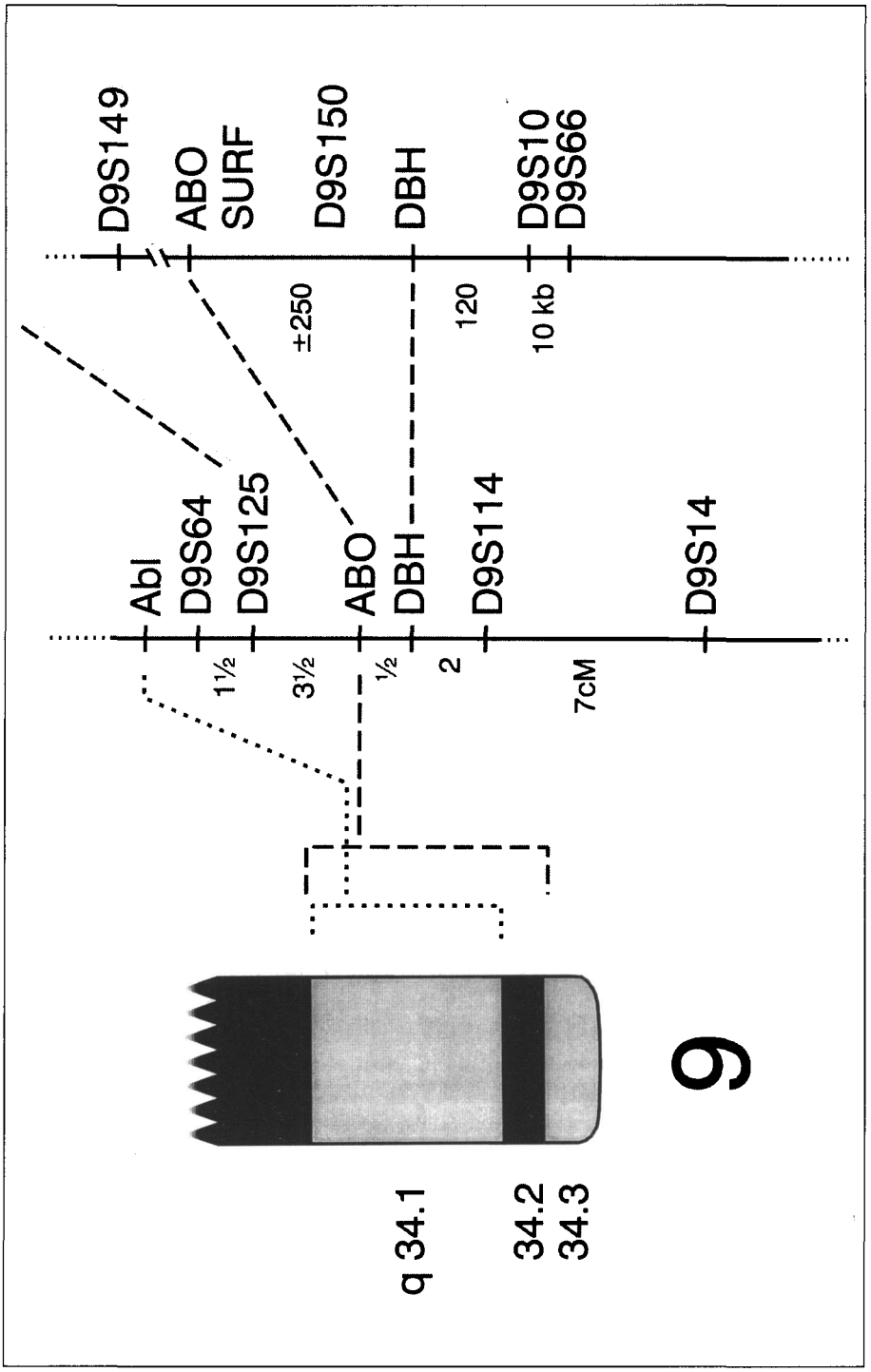

Fig. 1 - Candidate region of TSC1 (chromosome 9q34).

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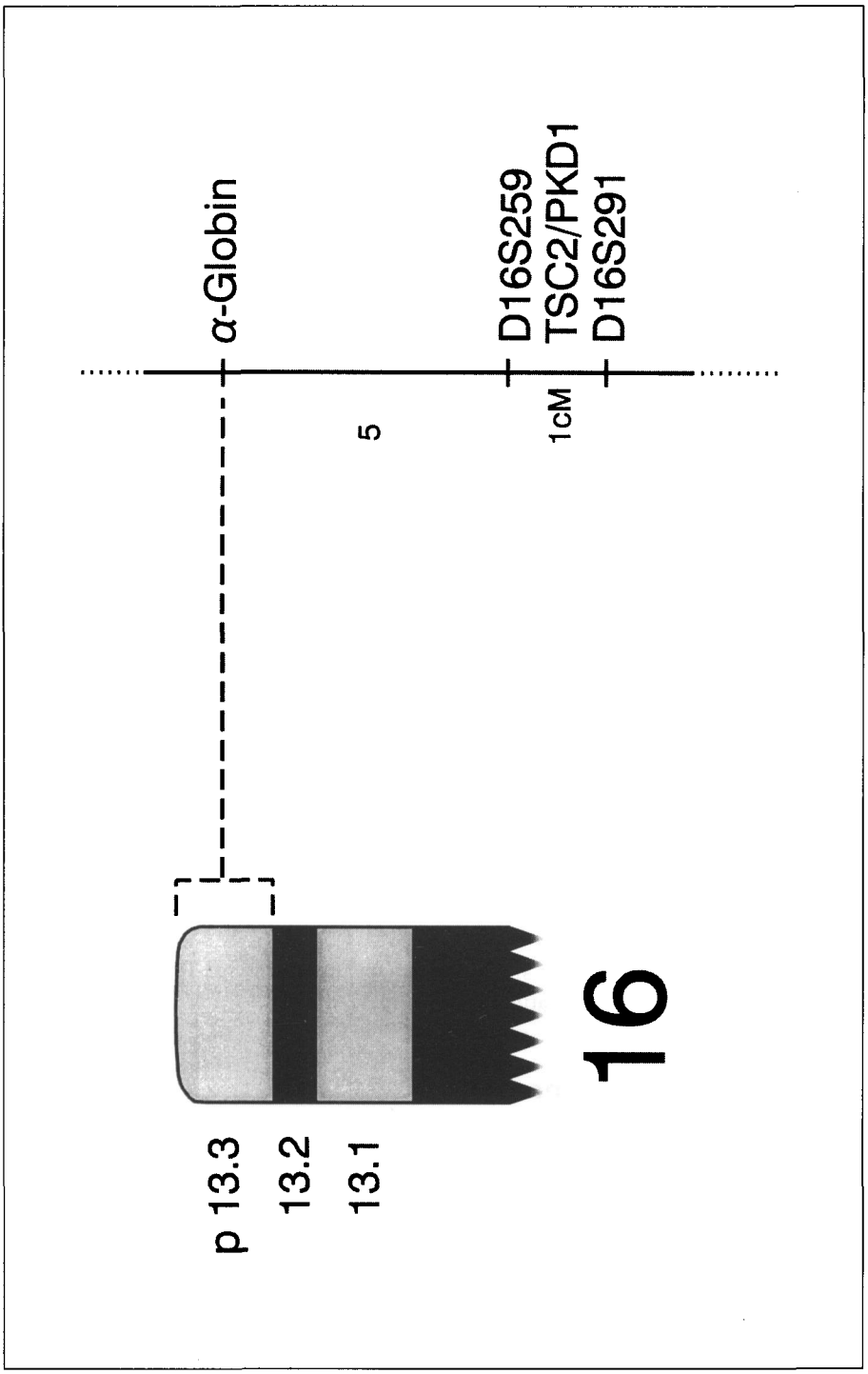

Fig. 2 - Candidate region of TSC2 (chromosome 16p13.3).

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The peak lod score for TSC2 was found at the position of marker D16S291 [20] (Fig. 2). This represents a clear illustration of the accuracy of the ICA, as, after its identification, the TSC2 gene was found to map at a physical distance of less than 200 kb from this marker [28]. The identification of the TSC2 gene followed the example of the majority of disease genes identified by means of positional cloning: a candidate region defined by genetic linkage as well as by chromosomal aberrations in patients.

Identification of the TSC2 gene

Linkage analysis had designated an estimated 1.5-Mb area on chromosome 16p13.3 as the candidate region for TSC2 [19]. Subsequently, a terminal chromosome 16p deletion in a patient with ATR-16 syndrome, but without any signs of TSC, and an unbalanced translocation resulting in monosomy of 16p13.3-pter in a TSC patient directed the search for the gene to the most proximal part of the candidate region. The family segregating the (16;22) (p13.3;q11.21) translocation has been crucial to the identification of the ADPKD gene as well, as the two carriers of the balanced translocation were diagnosed with ADPKD (Fig. 3), suggesting disruption of the gene by the translocation breakpoint.

The proximal candidate region was screened for large rearrangements in TSC patients' DNA by pulsed-field gel electrophoresis (PFGE) [28]. Five constitutional deletions were found and mapped to a 120-kb segment. Cosmid walking resulted in a series of overlapping clones spanning this segment. Cosmid-derived probes were used to screen cDNA libraries and four transcripts were identified [Fig. 4]. Three of the corresponding genes could be excluded as candidates for the TSC gene, because they were not involved in all of the PFGE-detected deletions. One of these genes encoding a large (15 kb) ubiquitously expressed transcript was later found to be disrupted by the t(16;22) translocation and identified as the gene involved in ADPKD [29]. The remaining gene mapped into all of the deletions. It was shown to be affected by several intragenic deletions in TSC patients, including a de novo case, and showed reduced levels of the normal 5.5-kb transcript in the affected members of a chromosome-16-linked family. Therefore, this gene was designated the TSC2 gene [28]. TSC2 is widely expressed in human tissues and showed conservation in higher vertebrates. A search for sequence homologies at the protein level revealed a region of similarity between the predicted 198-kD gene product "tuberin" and the GTPase-activating protein rap1GAP [28].

Mutations in the TSC2 gene

The identification of the TSC2 gene relied on the detection of an unbalanced translocation deleting the gene-containing region of chromosome 16, six interstitial deletions sized between 6 and 75 kb and affecting more than one coding sequence, and four small (1–5 kb) entirely intragenic deletions [28]. The volume of available patient material was large, as samples from 255 unrelated patients had been analyzed. Two main conclusions can be drawn from these initial results.

First, all of these mutations are inactivating. This is in line with other observations made in tumors of TSC patients, which showed allelic loss at (the normal chromosome)

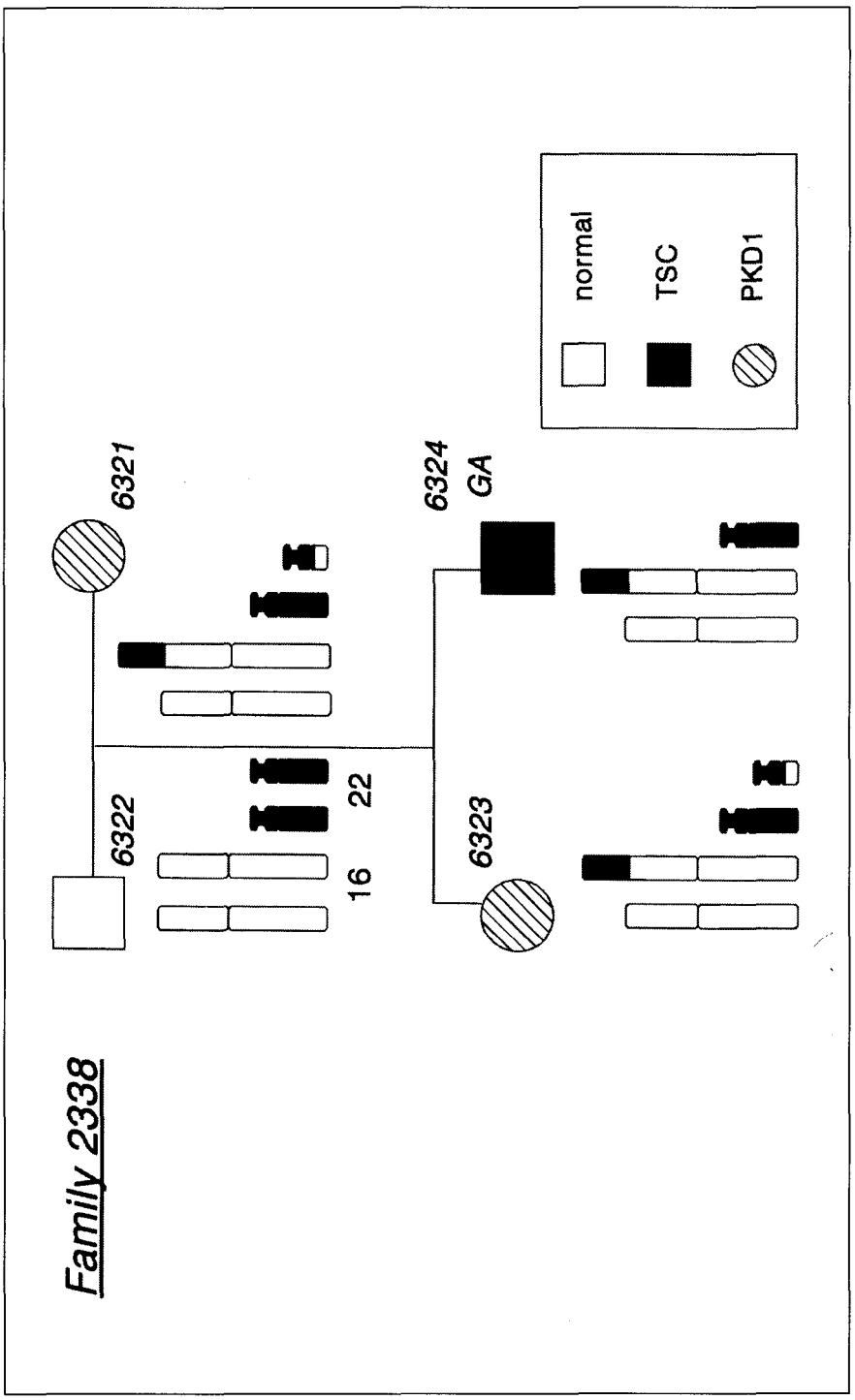

Fig. 3 - Pedigree of family 2338, which segregates a t(16;22) (p13.3;q11.21). PKD1 occurs in the carriers of the balanced translocation; TSC occurs only in conjunction with the unbalanced form of the translocation resulting in monosomy of 16p13.3 and 22q11.21-22pter.

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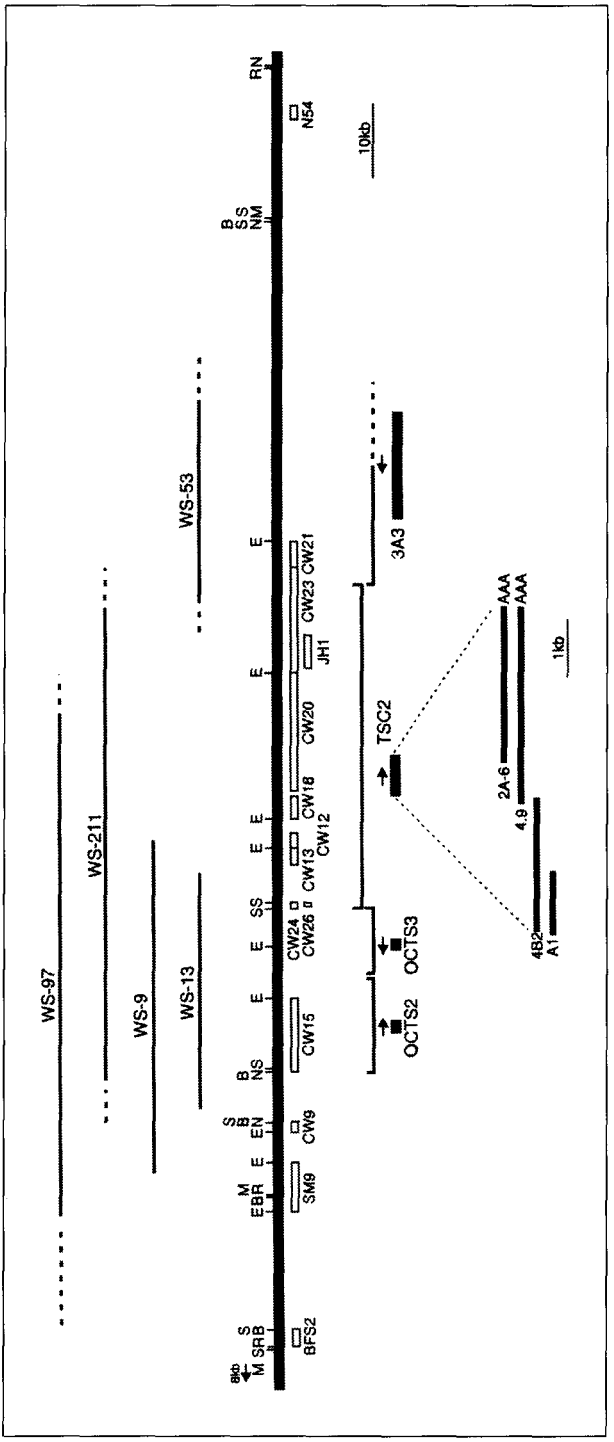

Fig. 4 - Map of the TSC area of chromosome 16. Restriction enzyme sites and genomic probes (open boxes) are indicated. TSC-associated deletions (WS-No) are shown. Below are the four genes identified in the area. TSC2 is disrupted by all deletions. 3A3 appeared to be disrupted by the translocation breakpoint (Fig. 3) of family 2338. [Adapted from ref. 28.]

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16p [30]. This was interpreted as a second hit affecting a tumor suppressor gene with a constitutional (inactivating) mutation. Since allelic loss has also been found in the chromosome 9q34 region which harbors the TSC1 gene [22, 23], the search for inactivating mutations like large deletions may also be instrumental in identifying the TSC1 gene.

The second conclusion is that there is a paucity of large rearrangements and thus easily detectable mutations affecting the TSC2 gene. The search for point mutations will be very time consuming, when performed at the DNA level, as the number of exons is now thought to be 40 [Nellist, et al., unpubl. results]. A comparable situation exists in NFI, a gene with a similar degree of genomic complexity and an as yet low proportion of readily detectable mutations [31]. An encouraging development in the detection of NFI mutations is the application of the protein truncation test, which seems to detect NFI gene mutations at a high rate [32, 33]. This method, which relies on the detection of truncated in vitro translation products reflecting translational stops, and other transcript-based techniques may be efficient instruments in the search for TSC2 mutations.

As with any newly discovered disease gene, the question arises whether correlations can be made between genotype and phenotype. This question is particularly challenging in view of the extreme phenotypic variation observed in TSC. Since extensive clinical variation can also be found within families, it is immediately apparent that the nature of the gene mutations cannot be the only explanation. However, even with the limited number of mutations known to date, some remarks can be made.

Brook-Carter et al. [34] studied 6 TSC patients who showed grossly enlarged polycystic kidneys within the first months of life. Although renal involvement is a common feature in TSC, the severity and age of onset in these patients were unusual. All of these patients were shown to have deletions affecting both the TSC2 and the adjacent gene, which is involved in ADPKD. Apparently, the renal aspect of this particular phenotype is determined by the inactivation of the PKD1 gene.

Recently, an individual has been identified with ambiguous signs of TSC and a son with a definitive diagnosis of the disease. The intragenic TSC2 deletion detected in the son's leukocyte DNA was also present in the father's DNA, but only in a proportion of the cells [35] (Fig. 5). It was proposed that the somatic mosaicism observed in the father may explain the phenotypic difference between father and son. Since TSC is characterized by a high frequency of new mutations, the identification of other cases of somatic or germline mosaicism can be anticipated. Some cases of intrafamilial clinical variation and apparent nonpenetrance are likely to be related to these phenomena.

TSC gene functions: possible clues from the TSC2 product tuberin

Allelic losses in TSC-related tumors have been observed in both chromosomal regions known to harbor a TSC gene [22, 23, 30]. This implies that at least part of the functions of the respective gene products may be growth suppression. A tumor suppression function of (the rat homologue of TSC2 seems especially pronounced in the Eker rat model of dominantly inherited cancer. The Eker rat develops specific malignancies with a predominant occurrence of renal tumors and was shown to have a constitutional insertion in the 3' part of the gene [36, 37]. Moreover, somatic loss of the wild-type allele has been

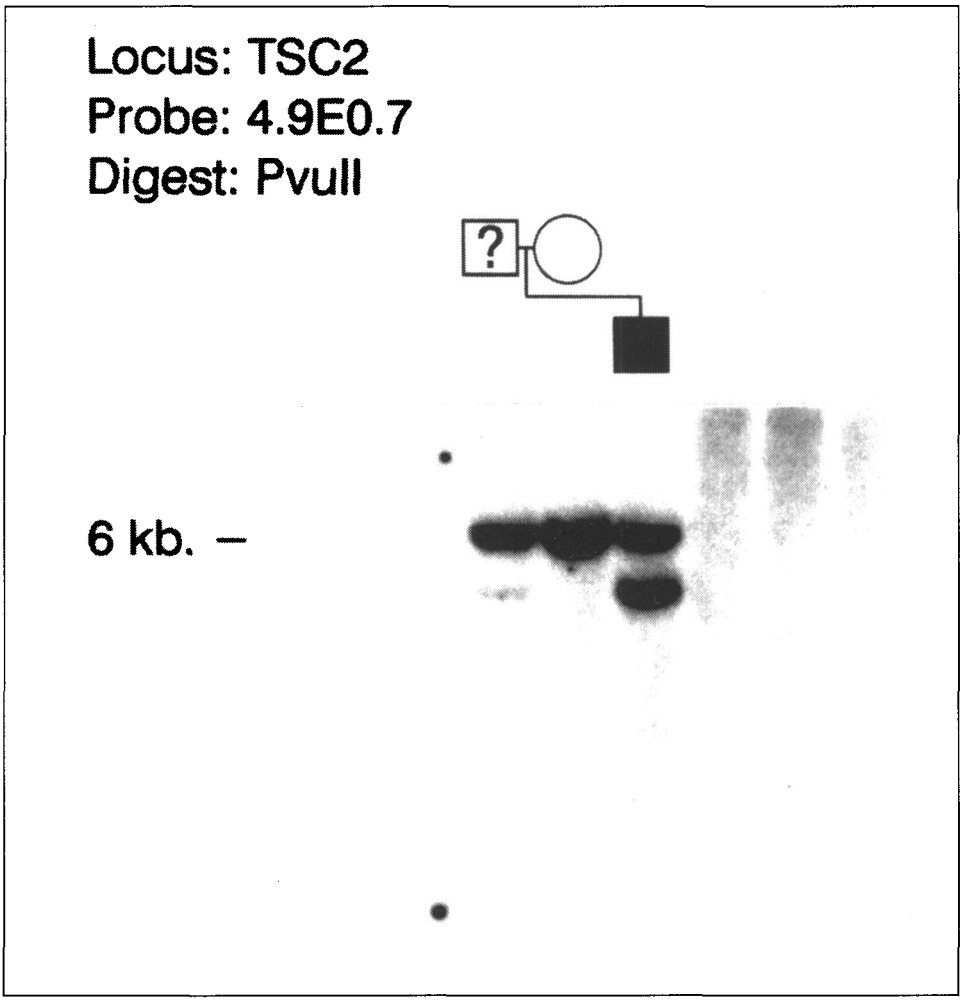

Fig. 5 - Leukocyte DNA probed with a TSC2 cDNA fragment. The son has TSC and his DNA shows a 1.5-kb deletion. The father has only ambiguous signs of TSC; the aberrant band has reduced intensity, reflecting mosaicism.

demonstrated in tumors. The Eker rat phenotype seems to be dramatically different from human TSC because of the absence of apparent skin manifestations and neurological abnormalities, and the invariably malignant nature of renal and other lesions [37, 38]. This may be related to diversity in phenotypes between species or to the characteristics of the Eker mutation. Apparently, the Eker rat would not be suitable as an animal model for the clinical spectrum of TSC, but it certainly constitutes a valuable model for cell-type-specific carcinogenesis.

Interestingly, the insertion in the Eker mutation has been reported to result in an aberrant transcript lacking the GAP-related domain. This small region of homology with

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rap1GAP might implicate tuberin in the regulation of cell proliferation and differentiation, as rap1 is a member of a group of GTPases known to be involved in these processes. At the moment, these considerations are necessarily theoretical, but in vitro translation of tuberin using full-length cDNA constructs will provide opportunities to address this issue experimentally.

Cruciarl to our understanding of the function of tuberin will be knowledge on the subcellular localization and the tissue distribution of the protein in normal and affected individuals. To analyze this, several groups are in the process of generating antibodies, both against synthetic peptides and fusion proteins corresponding to the entire coding region of the gene.

It is intriguing that, with the possible exception of a higher frequency of ungual fibromas in chromosome-9-linked families [39], no consistent clinical differences have been discovered between TSC1- and TSC2-type families. The TSC1 and TSC2 gene products are therefore presumed to perform complementary rather than equivalent functions. The gene products possibly interact as subunits of a heteromer, as receptor and ligand or as components of a common pathway. Insight into the normal and impaired function of tuberin may be a significant step towards an understanding of the locus heterogeneity.

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