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Linkage of an important gene locus for tuberous sclerosis to a chromosome 16 marker for polycystic kidney disease

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Tuberous sclerosis complex (TSC) is an autosomal dominant disorder of unknown aetiology that affects numerous body systems including skin, brain and kidneys. Some TSC has been linked to chromosome 9, additional TSC genes on chromosomes 11 and 12 have been proposed, but the majority of TSC families remain unlinked. Using TSC families in which data had excluded linkage to chromosome 9, we failed to detect linkage with loci on chromosomes 11, 12 and others. One marker examined was *D16S283*, the closest locus on the proximal side of the polycystic kidney disease type 1 (PKD1) gene. Linkage between TSC and *D16S283* demonstrated a lod score of 9.50 at $\Theta = 0.02$ with one family independently presenting a lod score of 4.44 at $\Theta = 0.05$. These data reveal an important TSC locus near the region of PKD1 on chromosome 16p13.

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder affecting numerous body systems, especially the brain, skin and kidneys¹. It is characterized by hamartomas (benign overgrowths, predominantly of a cell type or tissue type that normally occurs in the organ) and hamartias (developmental abnormalities of tissue combination) with resulting clinical symptoms ranging from benign hypopigmented macules (HMs) of the skin to profound mental retardation with intractable seizures. The estimated prevalence of TSC is 1 per 10,000 population² which is similar to that of Huntington's disease but less common than neurofibromatosis type 1, at 1 per 4,000 (ref. 3).

Despite years of study, the underlying defect in TSC has not been elucidated. In an effort to gain further understanding by means of a new approach, genetic linkage studies were undertaken by a number of research groups. In 1987, Fryer *et al.*⁴ demonstrated genetic linkage of TSC to the ABO blood group at chromosome 9q34. A second report confirmed linkage to this region⁵. However, subsequent studies by several investigators failed to support this linkage, thereby making interpretation of the initial localization difficult⁶⁻⁸. In 1990, linkage of TSC to a locus on chromosome 11q was suggested⁹. To resolve this apparent contradiction between loci on both chromosome 9 and 11, an international collaboration was established to analyse the joint data. The ensuing results¹⁰⁻¹² indicated that only a small proportion (approximately 33%) of families were linked to 9q, and suggested that other families might be linked to 11q. Also, reports from individual groups confirmed linkage of a subgroup of TSC families to chromosome 9 (refs 13-16). However, no

individual group has confirmed the chromosome 11 linkage^{17,18}. A further suggestion of linkage of some TSC families to chromosome 12q22-24 (ref. 19) has again not been independently confirmed^{17,18,20,21}. Also, several large, multigenerational TSC families clearly excluded linkage to all the proposed TSC loci^{17,20,21}; indicating that the majority of TSC families remained unlinked.

In order to identify the other locus for TSC, we assembled prospectively a set of five TSC families that had been studied for linkage to the previously-reported TSC loci, and, most importantly, were unlinked to chromosome 9. The families were investigated with DNA markers from the appropriate regions of chromosomes 9, 11 and 12, as well as numerous other marker loci throughout the genome. In addition, because of its potential relevance to TSC, we selected for study a locus, *D16S283*, in the region of the gene for autosomal-dominant polycystic kidney disease type 1 (PKD1)²². Herein, we report linkage of TSC to *D16S283* (SM7) at chromosome 16p13.

Sources of families and clinical evaluation

We obtained blood or lymphoblast cell lines for linkage analysis from 85 members (47 with TSC) of 5 multigenerational TSC families. These families were chosen because they were unlinked to the candidate locus on chromosome 9q34. Three of the families were previously described: family 437 (refs 7,13), family 16 (ref. 9) and family 18 (ref. 16). Family 437 is from New Zealand and the other four are from the United States. Two families are black (11, 1347), two are white (16, 18), and one family (437) is white with some Maori admixture. All patients diagnosed as TSC were either obligate heterozygotes

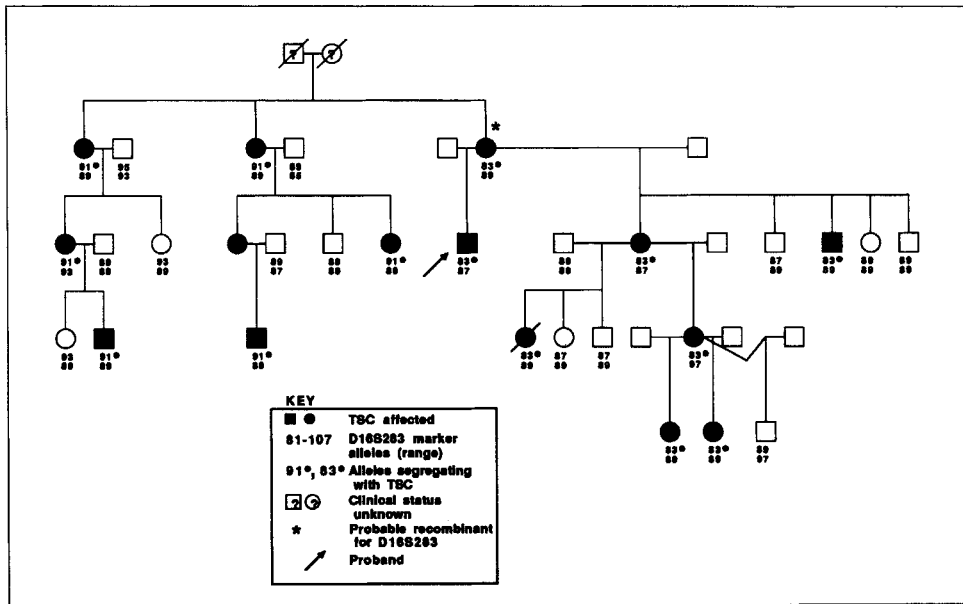


Fig. 1 Segregation of D16S283 alleles in family 437

(family 437, two of three affected individuals at the top of the pedigree; Fig. 1) or fulfilled the criteria of Gomez¹ in which they had at least one of the definitive diagnostic criteria or two of the presumptive diagnostic criteria.

We used the Gomez criteria to confirm the affected status of TSC, but these criteria were not designed to exclude the diagnosis. There were 17 genetically-at-risk but asymptomatic subjects from the five families. They were classified as unaffected after the following examinations: all 17 had negative skin examinations, however 9 of 9 unaffected members of family 437 did not have Wood's light exam, but the hypopigmented macules in the relatively dark-skinned affected individuals of family 437 were quite obvious without Wood's light. Five of 17 had negative retinal exams. Three of 17 had negative head CT scans and 1 of those also had a negative renal ultrasound. None of those coded as unaffected had mental retardation

or seizures. Two additional individuals at the top of the pedigree in family 18 were classified as unknown with respect to TSC because they were not available for examination. No evidence of nonpaternity was seen in the multiallelic markers used in this study.

Exclusion from chromosomes 9, 11 and 12

For chromosome 9, all families provided substantially negative lod scores for D9S66; two families had slightly positive lod scores for ASS. The chromosome 9 candidate region, the boundaries of which were recently defined¹⁷, was excluded by multipoint analysis in all families.

Except for family 16, the analysis of the chromosome 11 locus D11S35 provided substantially negative lod scores. Another locus on chromosome 11, TYR, was uninformative in these families with the

exceptions of family 16 that was positive and family 437 that was significantly negative. The chromosome 11 locus haplotype D11S144/D11S351 gave significantly negative lod scores in family 437. With the exception of family 16, multipoint analysis (Fig. 3) again excluded the potential chromosome 11 candidate region. (Chromosome 9 and 11 two-point lod scores are not shown but are available upon request.)

Two families gave slightly positive lod scores for chromosome 12 loci: family 16 for D12S7 and family 1347 for IGF1. With those two exceptions, however, all of the other chromosome 12 loci that were genotyped in the five families provided substantially negative scores (Table 1). A multipoint analysis of this chromosome 12 region was not possible, as no map that includes these loci is available. However, examination of the two-point data and the chromosome 12 loci haplotypes indicated that it is unlikely that multipoint analysis of this region would result in significant linkage.

As part of a genomic screen to identify the major TSC locus, 115 loci distributed over 21 chromosomes were tested, and failed to show evidence for linkage to TSC (data not shown; available on request).

Linkage of TSC to D16S283

Because PKD1 has potential relevance to TSC by virtue of cystic kidneys, and because D16S283 is both a highly informative probe and also the closest known locus on the proximal side of the PKD1 gene²², it was tested as part of the collaborative screening. Tests for linkage gave positive lod scores for D16S283 in all five families (Table 2) with a maximum total lod score of 9.50 at a maximum recombination fraction (Θ) = 0.02, with a 95% confidence interval of 0.001-0.11.

Family 437 independently achieved a lod score of 4.44 at Θ = 0.05. In this family, a single putative crossover event occurred, most likely in the individual indicated by a star in Fig. 1 who segregates the 83 bp allele with her TSC offspring while her affected siblings segregate the 91 bp allele. No other crossovers were apparent in the data set.

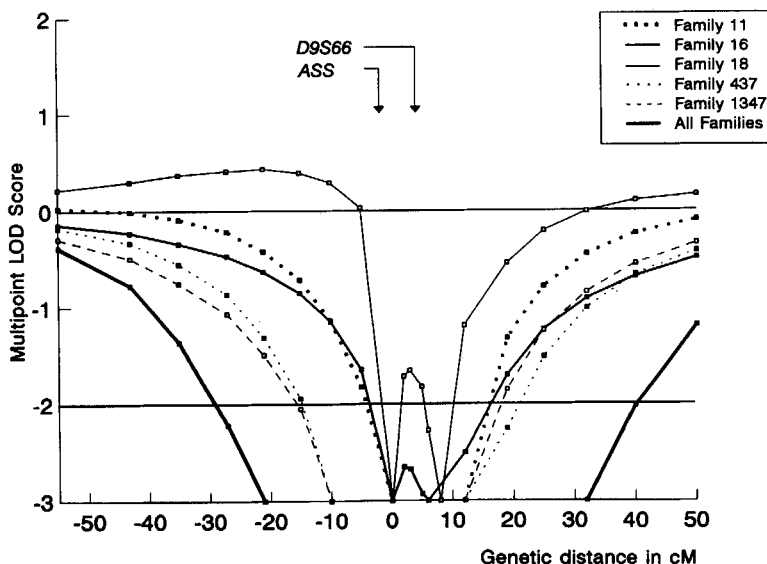


Fig. 2 Multilocus linkage analysis for chromosome 9 loci

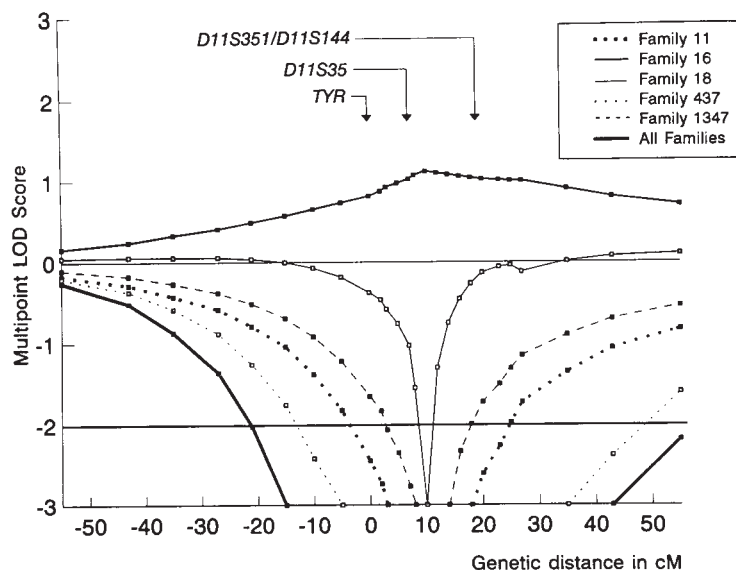


Fig. 3 Multilocus linkage analysis for chromosome 11 loci

Discussion

In light of the genetic heterogeneity of TSC, we felt it necessary not only to provide evidence for a significant linkage to chromosome 16, but also to investigate the alternative TSC loci. In this study, we chose five TSC families that clearly excluded linkage to the chromosome 9 candidate region. In selecting these families, negative lod scores for chromosomes 11 and 12 were considered less crucial as no independent group has confirmed these linkages. We believe our finding of linkage to chromosome 16 represents an important locus for TSC. Two lines of evidence support this contention. First, family 437 independently gives a peak lod score of 4.44 at $\Theta = 0.05$ with clear evidence for segregation of *D16S283* throughout the family (Fig. 1). Thus, the evidence from family 437 alone supports linkage of TSC to chromosome 16p13. Second, although not all families independently present significant evidence for linkage, all families were consistently positive for *D16S283*. (Family 16 also showed slightly positive lod scores for both chromosome 12 (Table 1) and chromosome 11 (*D11S35*: $z(\Theta)=1.13$, $\Theta=0.0$). It is therefore difficult to determine whether this family is truly one of the chromosome 16 linked group.) Therefore, this study provides strong support for the designation of chromosome 16 as the site of a locus for TSC.

D16S283 is the closest marker on the proximal side of the *PKD1* gene²², and, although it may be entirely fortuitous, this localization raises the question of a relationship of TSC to *PKD1*. Polycystic kidneys are present in a large number of people who have TSC, and are a recognized cause of renal failure and death in TSC²³. There have been instances in which a patient was diagnosed as having *PKD1* only to discover later that other members of the family had TSC or that the patient with polycystic kidneys actually had manifestations of TSC²⁴. Although they can be differentiated by their histologic features^{24,25}, the renal cysts of *PKD1* and TSC are clinically and radiologically indistinguishable. Several possibilities are raised by these observations: 1) TSC and *PKD1* may represent contiguous gene syndromes; 2) TSC and *PKD1* may be allelic variants; or 3) their genes may reside in a region affected by a dynamic mutation²⁶ similar to the triplet repeats in three other well-known neurologic syndromes: myotonic dystrophy²⁷⁻²⁹, fragile X syndrome (*FMR-1*)³⁰ and X-linked bulbospinal atrophy³¹.

Some of these issues may be clarified by fine mapping of the chromosome 16 TSC locus. For instance, DNA markers that flank *PKD1*³² might be used to determine if recombinant events such as the one in family 437 (Fig. 1) exclude TSC from the *PKD1* region. Furthermore, the area surrounding the *PKD1* gene has been extensively studied³², and similar techniques can be exploited to localize the segment of DNA that harbours the TSC gene on chromosome 16.

As a first step in attempting to localize the chromosome 16 TSC region, and to provide an accurate estimate of locus heterogeneity, it will be necessary to evaluate additional TSC families for this linkage, including those previously reported as linked to chromosomes 9, 11 and 12. If the chromosome 16p13 TSC locus proves to represent a major TSC gene, then additional investigations to identify the gene could include assembly of long-range restriction maps using pulsed field gel electrophoresis; use of somatic-cell hybrids to order markers with respect to the TSC locus, and development of contigs from cosmid,

Fig. 3 Multilocus linkage analysis for chromosome 11 loci

Table 1 Chromosome 12 lod scores by family

| Family | TSC vs D12S7 | | | | | |
|--------|-------------------------------------|-------|-------|-------|-------|------|
| | Recombination fraction (θ) | | | | | |
| | 0.00 | 0.05 | 0.10 | 0.20 | 0.30 | 0.40 |
| 11 | -3.56 | -0.53 | -0.28 | -0.09 | -0.02 | 0.01 |
| 16 | 0.90 | 0.82 | 0.74 | 0.58 | 0.40 | 0.21 |
| 18 | -4.22 | -0.64 | -0.26 | 0.06 | 0.16 | 0.13 |
| 437 | -4.29 | -0.66 | -0.29 | 0.04 | 0.15 | 0.14 |
| 1347 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Total | -11.17 | -1.01 | -0.09 | 0.59 | 0.69 | 0.49 |

| Family | TSC vs D12S8 | | | | | |
|--------|-------------------------------------|-------|-------|-------|-------|-------|
| | Recombination fraction (θ) | | | | | |
| | 0.00 | 0.05 | 0.10 | 0.20 | 0.30 | 0.40 |
| 11 | -3.60 | -0.92 | -0.59 | -0.27 | -0.11 | -0.03 |
| 16 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 18 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 437 | -3.90 | -1.07 | -0.67 | -0.29 | -0.11 | -0.02 |
| 1347 | 0.20 | 0.25 | 0.28 | 0.27 | 0.22 | 0.13 |
| Total | -7.30 | -1.74 | -0.98 | -0.29 | 0.00 | 0.08 |

| Family | TSC vs PAH | | | | | |
|--------|-------------------------------------|-------|-------|-------|-------|-------|
| | Recombination fraction (θ) | | | | | |
| | 0.00 | 0.05 | 0.10 | 0.20 | 0.30 | 0.40 |
| 11 | -6.27 | -2.47 | -1.42 | -0.52 | -0.14 | 0.00 |
| 16 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 18 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 437 | -16.24 | -4.11 | -2.54 | -1.08 | -0.39 | -0.07 |
| 1347 | -3.50 | -0.45 | -0.13 | 0.13 | 0.19 | 0.14 |
| Total | -26.01 | -7.03 | -4.09 | -1.47 | -0.34 | 0.07 |

| Family | TSC vs IGF1 | | | | | |
|--------|-------------------------------------|-------|-------|-------|-------|-------|
| | Recombination fraction (θ) | | | | | |
| | 0.00 | 0.05 | 0.10 | 0.20 | 0.30 | 0.40 |
| 11 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 16 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 18 | -4.26 | -0.72 | -0.44 | -0.19 | -0.08 | -0.02 |
| 437 | -4.54 | -1.73 | -1.21 | -0.64 | -0.31 | -0.11 |
| 1347 | -2.40 | 0.32 | 0.50 | 0.56 | 0.46 | 0.27 |
| Total | -11.20 | -2.13 | -1.15 | -0.27 | 0.07 | 0.14 |

Table 2 D16S283 lod scores by family

| Family | TSC vs D16S283 Recombination fraction (θ) | | | | | | |
|--------|---|------|------|------|------|------|------|
| | 0.00 | 0.01 | 0.05 | 0.10 | 0.15 | 0.20 | 0.30 |
| 11 | 0.55 | 0.53 | 0.49 | 0.43 | 0.37 | 0.31 | 0.20 |
| 16 | 1.12 | 1.10 | 1.01 | 0.91 | 0.80 | 0.69 | 0.47 |
| 18 | 1.76 | 1.74 | 1.63 | 1.50 | 1.35 | 1.19 | 0.85 |
| 437 | 2.29 | 4.12 | 4.44 | 4.24 | 3.90 | 3.47 | 2.43 |
| 1347 | 1.94 | 1.91 | 1.78 | 1.62 | 1.45 | 1.27 | 0.88 |
| Total | 7.66 | 9.40 | 9.35 | 8.70 | 7.87 | 6.93 | 4.83 |

$z(\hat{\theta}) = 9.50$, $\theta = 0.02$, 95% confidence interval = 0.001-0.11

bacteriophage or yeast artificial chromosome libraries. When the region of the TSC gene has been narrowed sufficiently, the DNA of TSC patients can be compared with that of normal patients for variation in triplet repeats or other structural changes as well as being cloned and sequenced to enable detection of possible mutations. Therefore, based on the linkage of TSC to a locus in the region of the PKD1 gene, there are several lines of future investigations that may elucidate the genetic mechanism for TSC.

Methodology

DNA preparation and biochemical analysis. Family DNA was prepared as described¹³. Polymerase chain reaction (PCR) analysis was performed as described^{33,34}. This method used oligonucleotides that prime for DNA sequences that contain (CA)_n repeats^{33,36}.

PCR was performed with ³²P-labelled dCTP, and the products were electrophoresed on 6% or 6.5% polyacrylamide gels. Autoradiography allowed detection of differently-sized fragments resulting from variation in size of polynucleotide repeats. Restriction fragment length polymorphisms (RFLPs) were detected by autoradiography of Southern blots. ³²P-labelled DNA probes were hybridized to the genomic DNA of family members that was immobilized on Southern blots and then exposed to X-ray film as previously described¹³. For PCR detection of RFLPs, published sequences³⁷⁻⁴⁰ of oligonucleotides from both ends of a DNA sequence that contained a variable restriction-enzyme site were synthesized. These were used as primers for PCR amplification of family members' genomic DNA. The DNA products of PCR were digested with appropriate restriction endonucleases and electrophoresed on ethidium bromide-stained agarose gels. The products of digestion were viewed under ultraviolet light and scored for the presence or absence of the variable site.

Linkage analysis. TSC was assumed to be inherited as an autosomal dominant trait with incomplete penetrance. Based on clinical examination, penetrance was estimated at 90%⁷. Varying the penetrances, however, did not change the lod score results significantly (data not shown). Mutation was not included in the model.

Assuming $\theta_{\text{males}} = \theta_{\text{females}}$ at a variety of recombination fractions, two-point lod scores were calculated for each family using the MLINK subprogram of the computer package LINKAGE (version 4.9)⁴¹. The 95% confidence interval was determined by subtracting one lod score unit from the peak lod score⁴². Multipoint analyses were performed using the LINKMAP subprogram of the LINKAGE package⁴³. These analyses were done using sets of markers having a known order on chromosomes 9 (ref. 35) and 11 (ref. 44). For chromosome 9, the following markers were used: ASS and D9S66; for chromosome 11: TYR (pMEL34 or the PCR-derived RFLP), MCT128.1/CJ208, and D11S35. The multipoint lod scores were calculated as described previously^{10,13}.

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