Linkage investigation of three putative tuberous sclerosis determining loci on chromosomes 9q, 11q, and 12q

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Abstract

Previous linkage studies in tuberous sclerosis have implicated three disease determining loci at 9q, 11q, and 12q. We have collated phenotypic and genotypic data on 1622 members of 128 families with tuberous sclerosis in order to evaluate simultaneously the evidence for these putative loci. Affection status in the family members has been reassessed using uniform diagnostic criteria and genotypic data extensively checked before analysis under alternative models of locus heterogeneity. One tuberous sclerosis determining locus, accounting for approximately 50% of the families studied, has been found to map in the region of *D9S10* on 9q34 but no evidence has been found to support the existence of major loci on 11q or 12q. A locus, or loci, elsewhere in the genome is likely to account for tuberous sclerosis in most non-chromosome 9 linked families.

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Genetic linkage studies in tuberous sclerosis (TSC) have been hampered by a relative paucity of large families, by variable expression and occasional non-penetrance of the causative genes, and, in particular, by locus heterogeneity. A combination of these factors is likely to account for the apparently discrepant conclusions reached by a number of previous linkage investigations.¹⁻⁴

Linkage between *TSC* and the ABO blood group locus on distal 9q was first indicated by a study of 19 UK families generating a lod score of 3.85 at $\theta=0$.¹ In one family uninformative for ABO, a lod score of 1.2 at $\theta=0$ was obtained with *AK1*, which maps to the same region. Further support for this assignment came from study of an RFLP at the Abelson oncogene locus (*ABL*) in a subset of these families. A lod score of 3.18 at $\theta=0$ was

observed.⁵ In contrast, two later multifamily studies obtained negative scores between *TSC* and markers from 9q34.^{3,6} The authors of the latter studies suggested non-linkage or locus heterogeneity as possible explanations for the discrepancy between their own findings and those of the earlier studies, but did not obtain significant evidence for locus heterogeneity. Soon two family types, those linked and those not linked to *ABL*, were clearly documented and *D9S16* and *D9S10* were identified as probable flanking markers for the *ABL* linked locus, *TSC1*.⁷ Assignment of the non-*ABL* linked *TSC* gene to 11q14-23 was proposed after a study of 15 families which generated positive lod scores with five markers from 11q.² Assuming 90% penetrance, lods of 3.26 ($\theta=0.08$) and 2.88 ($\theta=0$) were obtained with *MCT128.1* (*D11S144*) and *TYR* respectively. Analysis with the USERM9 subprogram from MENDEL⁸ showed no evidence of locus heterogeneity and the lods were calculated assuming homogeneity.

Janssen *et al*⁹ used an alternative approach (the 'imaginary chromosome method') for synchronous evaluation of the putative *TSC* loci on 9q and 11q. Nine families were studied with multiple markers from both chromosomal regions. Two *TSC* loci, one in each region, gave a significantly better fit than one locus alone ($p=0.044$) and maximum likelihood estimates for the positions of *TSC1* and *TSC2* were just proximal to *ABL* and at L7 (*D11S29*) respectively. No evidence for a family type unlinked to either region was found.

Following the 10th International Human Gene Mapping Conference a consortium of groups undertaking linkage studies in *TSC* was formed. Linkage data for markers on 9q and 11q were shared and analysed using alternative applications of the HOMOG programs^{10,11} and new two point approaches.¹² All analyses reached similar conclusions; a *TSC* locus could clearly be shown on 9q and the

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evidence for locus heterogeneity was overwhelming. Positioning of *TSC1* in relation to 9q34 markers was imprecise, reflecting uncertainty in classification of families as *TSC1* or *TSC2* type. Localisation of *TSC1* between *ORM* and *D9S14* was consistent with the findings of the alternative analytical approaches. Evidence for a locus on chromosome 11 was considered to be strong, although flanking markers could not be identified.

A more recent investigation of 22 families with multiple markers from 9q and 11q confirmed locus heterogeneity with one locus (*TSC1*) on distal 9q.¹³ Recombination events in families likely to be chromosome 9 linked suggested localisation of *TSC1* to the interval *ASS-D9S7*. No evidence for a locus on 11q could be established in the data as a whole or in a subset of families unlikely to be chromosome 9 linked.

The report of a third putative TSC locus on 12q, identified through a disease associated translocation¹⁴ and linked to *PAH*,⁴ was therefore of considerable interest. In 15 families a lod score of 4.33 at $\theta=0$ was observed between *TSC* and *PAH*. HOMOG analysis using multipoint lod scores derived with *D12S7*, *S8*, and *PAH* showed no evidence of heterogeneity and cumulative lods observed with markers from 9q and 11q were substantially negative.

Previous linkage studies have therefore implicated three loci in TSC, but have not evaluated these together. We set out to assess the evidence for the putative loci using a large and rigorously checked data set. This was collected from the centres constituting the Tuberous Sclerosis Collaborative Group and included the data subsets on which the previously suggested assignments were based.

Materials and methods

Both genotypic and phenotypic data were available for 1622 members of 128 multiplex TSC families. Much of the family material has been documented in previous publications and it is considered in more detail in the accompanying paper.¹⁵

PHENOTYPIC DATA

Clinical and radiological information which could be used to classify affection status was collected on all family members. Modified linkage format pedigree files were used for this purpose. Age at assessment and presence, absence, or uncertainty with regard to the following diagnostic criteria were recorded: adenoma sebaceum, periungual fibromata, shagreen patches, hypomelanotic macules, fibrous forehead plaques, retinal phakomata, typical periventricular calcification on brain CT scan or MRI scan, cortical tubers on MRI scan or at necropsy, and renal angiomyolipomata or cysts detected radiologically or at necropsy. In addition, information on seizures, mental retardation, brain tumours, and any unusual features was recorded.

The data were reviewed by one of us (JRS) and used to classify each subject into one of

four liability classes: AFFECTED, where a definitive diagnosis could be made according to the criteria of Gomez, UNKNOWN 1 where no information was available, where incomplete but normal clinical information was available, or where findings of uncertain clinical significance had been made (for example, solitary renal cyst, seizures, or mental retardation, but no other positive signs), UNKNOWN 2 where complete clinical examination proved normal but where radiological workup was incomplete, and NORMAL where full clinical and radiological workup, including Wood's light examination, ophthalmoscopy, brain CT or MRI scan, and renal CT or ultrasound scan, showed no evidence of TSC in a family member over 16 years of age.

Liability classes corresponding to a penetrance of 95%, a phenocopy rate of 2%, and a conservative estimate of clinical gene expression (that is, disease apparent without radiological investigation) of 67% were used in the analysis. The liability classes were selected to minimise mapping errors consequent on misinterpretation of clinical or radiological signs.

GENOTYPIC DATA

A modified version of MLINK (provided by L A Sandkuijl) was used to identify definite and probable double recombinants by examining all possible combinations of three markers in the same chromosomal region in each family. Those with probable ($>10:1$) double recombinants were retyped (in each case with resolution to a zero or single recombinant solution) or excluded from the analysis. Recombination fractions were calculated for pairs of markers in each chromosomal region for each group and these were found to be consistent with published data and did not show significant intergroup variation (data not shown).

A database of available marker data was compiled and marker loci were selected for inclusion in the analysis on the basis of three criteria: adequate coverage of the proposed candidate regions, comprehensive typing by the contributing groups, and high informativity. Where a family was typed for two or more polymorphic systems at a single marker locus the most informative system in that family was selected.

The marker loci used in the analysis and their map positions, taken from published data,¹⁶⁻²³ are shown in fig 1.

Allele frequencies were calculated (from founder members and spouses only) for the data of each group and for the data as a whole. They did not differ significantly from published frequencies²⁴ which were used in linkage analysis. Dinucleotide repeat markers were recoded to 4/5 alleles without loss of linkage information.

LINKAGE ANALYSIS

For each family MLINK was used to calculate multipoint lod scores at specified positions across maps of the three candidate regions.

CHROMOSOME 9

Cen - GSN - AK1 - ASS - ABL - S10 - S14 - S7* - Tel
 0.09 0.05 0.01 0.06 0.06 0.14

CHROMOSOME 11

Cen - S84 - S35 - S144 - S351 - S29 - PBGD - S147 - Tel
 0.09 0.15 0 0.04 0.04 0.04

CHROMOSOME 12

Cen - S8 - S7 - PAH
 0.19 0.16

Figure 1 Genetic maps of the chromosomal regions analysed. Genetic distances expressed as sex averaged θ . *D9S7 data were omitted from the analysis.

There were 45, 40, and 43 positions at 1 cM intervals between the markers on chromosomes 9, 11, and 12 respectively. For each family these positions were defined by calculating the distances to and from the nearest informative proximal and distal flanking markers using Haldane's mapping function.²⁵ Therefore, markers uninformative in a given family were not included in the analysis. Lod scores were also calculated at four 'off map' positions at $\theta = 0.1, 0.2, 0.3$, and 0.4 either side of each region. Interference was assumed to be absent and male and female recombination frequencies equal. The mutation rate was set at 2.5×10^{-5} and the disease gene frequency at 1×10^{-4} .

In summary, one multipoint run was therefore used to calculate the lod score at one position in one family. Arrays of lod scores calculated at identical positions independent of the pattern of informativity formed the input

files for heterogeneity tests using the HOMOG programs.

Because the large number of haplotypes generated in highly informative families exceeded the capacity of PCs, lod scores were computed, where necessary, on a DEC 5830 and on Sun workstations using several overlapping multipoint analyses as described by Ott.²⁶

HETEROGENEITY ANALYSIS

Sets of lod scores for different chromosomal regions can be analysed synchronously using the HOMOG programs,²⁷ by their combination into a single array. This method has been termed the 'imaginary chromosome approach'⁹ and is suitable for simultaneous mapping of two or more loci.

The programs HOMOG, HOMOG2, and HOMOG3 were used to evaluate the evidence for locus heterogeneity and for one, two, or three TSC loci in the tested regions. Analyses were performed for all combinations of chromosomal regions for the data as a whole and the subsets of data contributed by each centre were also submitted to a more restricted series of analyses.

The HOMOG programs assume equal penetrance, mutation rate, and gene frequency for the different disease determining loci.

Results

INFORMATIVITY IN THE TESTED REGIONS

The number of families informative at each locus analysed and the number of families informative for each chromosomal region as a whole are indicated in the table.

Number of informative families at each marker locus and in each chromosomal region (totals).

Chromosome 9		Chromosome 11		Chromosome 12	
Marker	Families	Marker	Families	Marker	Families
GSN	38	S84	38	S8	34
AK1	28	S35	60	S7	62
ASS	103	S144	73	PAH	49
ABL	72	S351	62		
S10	79	S29	33		
S14	21	PBGD	47		
		S147	41		
Total	106		101		62

Figure 2 Scheme showing likelihoods for models of heterogeneity incorporating TSC loci on all combinations of chromosomes 9, 11, and 12. Refer to text for details.

EVIDENCE FOR HETEROGENEITY AND FOR TSC LOCI ON CHROMOSOMES 9, 11, AND 12

The relative likelihoods for models of heterogeneity including TSC loci in all possible combinations of the chromosome 9, 11, and 12 regions are summarised in fig 2.

Lod scores at the points of the triangle were calculated using data for the indicated single chromosomal region alone. At the points of the triangle Lod(1) indicates the logarithm of the odds computed under homogeneity and Lod(2) that computed under heterogeneity using the HOMOG program (that is, assuming that there is a proportion of unlinked families in which the disease locus maps to an unspecified alternative region). There is significant evidence for a TSC locus on chromosome 9, but not on chromosomes 11 or 12. The chromosome 9 data clearly support heterogeneity (chromosome 9 and non-9 only model), Lod(2) being 3.31 lod units above Lod(1).

Lod scores shown along the sides of the triangle were computed using the data for the corresponding two chromosomal regions. These were calculated under heterogeneity allowing for two and three loci (Lod(2) and Lod(3)) using the HOMOG2 and HOMOG3 programs respectively. HOMOG2 allows for

the presence of two loci on the map. Alternatively, one of the loci may be placed 'off map' (that is one of the loci is unlinked to either region) and here the analysis will yield results identical to those of the HOMOG program. Only lods calculated from data including chromosome 9 are significant and the data for chromosomes 11 and 12 do not significantly improve the lod score over that for the chromosome 9 and non-9 only model. Minor differences in lod scores between analyses not reaching significance may reflect variation in θ values associated with peak lod scores in unlinked families.

Analysing all three chromosomal regions synchronously using HOMOG3 the lod (indicated as Lod (3) inside the triangle) is not significantly higher than that for the chromosome 9 non-9 only model.

In no analysis was a locus positioned on the chromosome 11 map. Analyses of the chromosome 12 data alone, the combined chromosome 11 and 12 data, and the HOMOG3 analysis of the chromosomes 9, 11, and 12 data, while not reaching significance over the chromosome 9 non-9 only model did place one locus between *D12S7* and *PAH*. The corresponding α values were 10 to 20%.

Evaluation of the data from the individual contributing centres (analyses not shown) showed that the data from all except Erlangen were consistent with the conclusions drawn from the data set as a whole. Interestingly, data sets previously found to support a locus on chromosome 11 did not do so in this analysis, but did support the chromosome 9 locus. This possibly reflects gain of information from recent marker studies, or loss of information owing to our restrictive diagnostic classification. On analysis of all families the main evidence for a locus on chromosome 12 (which did not reach significance) came from the Erlangen families. When analysed separately the Erlangen data favoured a locus on chromosome 12 (at *D12S7*) in 95% of families (lod score = 2.88). When analysed with the locations and α values obtained in the combined

analysis, we observed that the Erlangen families contributed only 0.88 to the overall Lod(3) score.

In summary, there is significant evidence for a TSC locus in the tested region of chromosome 9 (*TSC1*), but not in the tested regions of chromosomes 11 or 12. Although locus heterogeneity exists there is no evidence that a major non-chromosome 9 locus maps to the other regions studied.

THE PROPORTION OF FAMILIES SEGREGATING FOR A *TSC1* MUTATION

In the simplest model of heterogeneity reaching significance, chromosome 9 and non-9 only, the maximum likelihood estimate of α (proportion of chromosome 9 linked families) is 51%. In the other models of heterogeneity reaching significance the maximum likelihood estimates of α varied between 45% and 55%.

LOCALISATION OF *TSC1*

All HOMOG and HOMOG2 analyses incorporating the chromosome 9 data identify the maximum likelihood position for *TSC1* as 1 cM proximal to *D9S10*. Two of three HOMOG3 analyses also identify this position and one (chromosome 9 and 11 data only) identifies *D9S10*.

The POINT4 program was used to estimate the support region for *TSC1* based on a difference of 1 unit of $\log_{10}$ likelihood from maximum under all sets of parameter values (α_1 , α_2 , and disease gene locations, x_1 , x_2 , and x_3). This identified a region extending from 1 cM distal to *ABL* to 4 cM distal to *D9S10* (fig 1).

Under analysis of locus heterogeneity only one combination of α and map location (or θ) will typically be associated with the maximum likelihood solution. However, various combinations of α and map location will be associated with equal, but lower, likelihoods. Fig 3 shows the point of maximum likelihood ($Z_{\max} = 13.76$) and contours corresponding to ($Z_{\max} - 1$), ($Z_{\max} - 2$), ($Z_{\max} - 3$), and ($Z_{\max} - 4$) lod units obtained on analysis of the chromosome 9 data for all families.

POSTERIOR PROBABILITIES OF CHROMOSOME 9 LINKAGE BY FAMILY

Fig 4 shows the distribution of posterior probabilities for families segregating a *TSC1* mutation as determined by HOMOG analysis of the data for chromosome 9, that is, with *TSC1* positioned 1 cM proximal to *D9S10*.

Discussion

Linkage analysis has proved to be a successful strategy for mapping disease genes in situations where the majority, or even all, subjects with the disease phenotype have mutations at a single locus.^{28,29} However, many genetic diseases are likely to reflect dysfunction of heterodimeric proteins, acceptor-receptor interaction or interruption of a metabolic pathway at one of a number of intermediary steps. Even

Figure 3 Possible α values (proportion of chromosome 9 linked families) and map locations for *TSC1* corresponding to $Z_{\max} - 1$, -2 , -3 , and -4 lod units.

Figure 4 Distribution of posterior probabilities for families being *TSC1* type.

mutations affecting entirely separate cellular or physiological systems may generate similar phenotypes. Locus heterogeneity, and its implications for linkage approaches, should therefore be anticipated. When large families are available for one or more of the contributing loci,³⁰ or when mutations at one of the loci are responsible for the majority of familial cases,³¹ it will generally be possible to map such a locus accurately. In contrast, when only small families are available, or when several loci exist, each being responsible for a small proportion of families, localisation of the respective loci using linkage analysis will be virtually impossible. In the accompanying paper¹⁵ we evaluate in more detail the average family size in the current data set, and its suitability for localisation of multiple TSC loci.

We have used an approach enabling evaluation of three putative TSC determining loci, on 9q, 11q, and 12q, in a single analysis. The possible existence of loci in these regions was supported by previously published investigations obtaining lod scores reaching conventional levels for significance.¹²⁴ Larger collaborative analyses had apparently confirmed the existence of the chromosome 9 locus (*TSC1*) and provided strong but not incontrovertible evidence for a second locus on 11q.¹⁰⁻¹² We chose to re-evaluate all clinical information and ignore all previously ascribed affection statuses. This resulted in changes in many families. We also sought to minimise typing errors by assessing marker-marker estimates of θ in the data of each contributing group, and by identifying and eliminating probable double recombinants.

Our analysis confirms the assignment of one gene determining TSC to 9q34, and indicates its most probable location as just proximal to *D9S10*. Mutations in this gene account for an estimated 45 to 55% of the 128 families studied. There is minimal support for the putative locus on 12q and none for the putative locus on 11q. While the possibility of very rare loci in any region can never be ruled out, it is clear

that a locus outside the tested chromosomal regions accounts for the majority of non-chromosome 9 linked families. Even the subsets of data which previously implicated loci at 11q and 12q did not give significant evidence to support their existence in this analysis. None of the analyses placed a TSC gene on the chromosome 11 map in any proportion of families and this situation was not changed by analysis of a more proximal subset of markers, *TYR-D11S84* (L A J Janssen, unpublished results). Some analyses mapped a small proportion of families to a position between *D12S7* and *PAH*, but the likelihoods obtained were not significant over an 'off map' localisation for all non-chromosome 9 families. The possibility of a rare chromosome 12 locus might usefully be clarified by improved clinical evaluation of the very few families hinting at linkage in this data set.

Localisation of the major non-chromosome 9 TSC gene will assist with linkage resolution of *TSC1*. Haplotype analysis in the few large chromosome 9 linked families available and the identification of key recombinant subjects should be particularly helpful in this regard. Even with the mapping of *TSC1* and *TSC2* to intervals of a few cM, the combination of locus heterogeneity and small mean family size will severely limit the application of linkage approaches in the diagnostic setting. For the majority of families this must await the isolation and characterisation of the TSC genes.

Note

It is not possible to provide lod scores for all families individually in a paper of this kind, but those interested may approach the first author for a computer disk containing these.

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