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# Loss of heterozygosity on chromosome 16p13.3 in hamartomas from tuberous sclerosis patients

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Tuberous sclerosis (TSC) is an autosomal dominant condition with characteristic skin lesions, mental handicap, seizures and the development of hamartomas in the brain, heart, kidneys and other organs. Linkage studies have shown locus heterogeneity with a TSC gene mapped to chromosome 9q34 and a second, recently identified on 16p13.3. We have analysed DNA markers in eight hamartomas and one tumour from TSC patients and found allele loss on 16p13.3 in three angiomyolipomas, one cardiac rhabdomyoma, one cortical tuber and one giant cell astrocytoma. We suggest that the TSC gene on 16p13.3 functions like a tumour suppressor gene, in accordance with Knudsen's hypothesis.

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Tuberous sclerosis (TSC) is an autosomal dominant condition characterized by tumour-like malformations (hamartomas) of the skin, brain, heart, kidney and other organs. The disease prevalence is estimated at 1 in 10,000, with two thirds of cases being sporadic and representing new dominant mutations. Half of patients are mentally retarded and most have seizures with one or more of the characteristic dermatological lesions: facial angiofibromas (adenoma sebaceum), hypopigmented macules (ash leaf patches), forehead plaques, shagreen patches and ungual fibromas<sup>1</sup>. There is wide variation in expression. Common hamartomas occurring in TSC include cortical tubers, subependymal nodules, retinal astrocytomas, renal angiomyolipomas and cardiac rhabdomyomas. Giant cell astrocytomas, which are essentially benign tumours, also occur.

A TSC gene was mapped to 9q34 in 1987<sup>2</sup>, and subsequently localized between flanking markers *D9S67* and *ASS*, representing a genetic interval of approximately 14 cM<sup>3</sup>. There is locus heterogeneity in TSC with many families not showing linkage to the 9q34 locus<sup>4</sup>. Other TSC loci have been suggested on chromosomes 11 and 12<sup>4,5</sup>. Recently, in TSC families not linked to chromosome 9q34, linkage was demonstrated to the marker *D16S283* (*SM7*), on chromosome 16p13.3<sup>6</sup>, and the gene, *TSC2*, subsequently identified<sup>7</sup>. It appears that the two loci on 9q34 and 16p13.3 account for most if not all of the cases of familial TSC<sup>3</sup>.

Another autosomal dominant condition with some resemblance to TSC is neurofibromatosis type 1 (NF1) characterized by benign tumours of peripheral nerves, pigmented skin lesions and retinal hamartomas. The gene for NF1 has been cloned and a somatic deletion of the

normal allele at this locus has been described in a neurofibrosarcoma from a familial case of NF1<sup>8</sup>. This gave rise to the suggestion that the NF1 gene acts as a tumour suppressor<sup>9</sup>. There has been speculation that the lesions in TSC might arise in a similar way<sup>10</sup>. We set out to examine DNA extracted from hamartomas and tumours from TSC patients to look for allele loss using markers from 9q34 and 16p13.3.

## Results

Six out of the nine lesions analysed showed allele loss for one or more markers on 16p13.3. Of the six renal angiomyolipomas studied, two showed loss of an allele at the marker *KG8* (Fig. 1a) and one showed almost complete loss of an allele at *D16S85* (3'HVR). Three angiomyolipomas did not show allele loss. The cardiac rhabdomyoma showed allele loss for *KG8*, but not for markers proximal to *KG8* (Fig. 1b). A cortical tuber from the same patient also showed loss of the same allele for *KG8*. The giant cell astrocytoma showed almost complete allele loss for *D16S309* (*MS205*) and *D16S85* (Fig. 1c, d). No allele loss was found for the chromosome 9 markers *ASS*, *D9S64* or *D9S66* which flank the TSC locus on 9q34 (data not shown). There was also no loss of heterozygosity for markers on chromosomes 3p, 11p, 11q or 12q chosen as controls. All but the last of these are regions which show allele loss in several tumour types including renal carcinomas (3p), Wilm's tumour (11p) and embryonal rhabdomyosarcomas (11p). 11q and 12q have been suggested as possible loci for a TSC locus.

The distribution of allele loss in the different lesions is shown in Table 1. *KG8* showed allele loss in all the lesions that were informative for this marker. As *KG8* was the



Fig.1 a KG8 PCR products analysed on 10% polyacrylamide gel, stained with ethidium bromide, demonstrating loss of heterozygosity and absence of heteroduplex formation in DNA from angiomyolipoma 3. Lanes 2 and 3, DNA from renal angiomyolipoma 3 showing a single 112 bp allele. Lane 1, Leucocyte DNA from the same patient showing 112 and 116 bp alleles. b, KG8 PCR products analysed on 10% polyacrylamide gel, stained with ethidium bromide, demonstrating loss of heterozygosity in DNA from the cardiac rhabdomyoma (lane 2). Lanes 1 and 3, DNA from spleen of the same patient showing 110 and 114 bp alleles. c, Southern blot probed with *D16S309*, showing significant loss of the upper allele in the giant cell astrocytoma. Lanes 2 and 4, DNA from the giant cell astrocytoma showing a strong 2.4 kb allele and a very faint 2.6 kb allele. Lanes 1 and 3, Leucocyte DNA from the same patient showing 2.4 and 2.6 kb alleles. d, Southern blot probed with *D16S85*, showing almost complete loss of the lower allele in the giant cell astrocytoma. Lanes 2 and 4, DNA from the giant cell astrocytoma showing a strong 3.8 kb allele, and a very faint 2.0 kb allele. Lanes 1 and 3, Leucocyte DNA from the same patient showing 3.8 and 2.0 kb alleles.

most distal of the dinucleotide repeat markers, we were unable to map allele loss distal to KG8 in those lesions where DNA could only be extracted from paraffin embedded tissue. *D16S291* was the proximal limit of allele loss in one angiomyolipoma and in the giant cell astrocytoma, cortical tuber and cardiac rhabdomyoma.

All of the allele losses were in lesions from sporadic cases of TSC. One patient, who showed allele loss in an angiomyolipoma (AML4), has an unaffected daughter. Typing of the daughter and her father showed that she had inherited the allele deleted in the angiomyolipoma, by implication the allele not associated with TSC (see Fig. 2).

### Discussion

We have described allele loss on chromosome 16p13.3 in

six of nine lesions from patients with TSC. In four of these samples, loss of heterozygosity was demonstrated for the marker KG8, which is only 30 kb from the newly cloned tuberous sclerosis gene<sup>7</sup>. We were unable to demonstrate allele loss for markers flanking the 9q34 TSC locus but could not exclude the possibility of a small deletion of this region.

The use of PCR-based dinucleotide repeat markers on DNA extracted from archival paraffin-embedded tissue had advantages and disadvantages. The technique permitted extraction of DNA from small homogeneous areas of the lesion. The quality of DNA extracted, however, only allowed amplification of small fragments of DNA and thus limited the markers that could be used to dinucleotide repeats where the PCR product is less than 250 bp in size. In the region of interest on 16p13.3, the most distal dinucleotide marker available was KG8, so that we were unable to map allele loss distal to this marker in the paraffin embedded tissue samples.

There are three reasons why we consider these findings are likely to have a bearing on the primary pathology of TSC. Firstly, the loss is occurring within the chromosomal region where a locus for TSC is known to lie. Secondly, the loss we describe is in hamartomatous or benign lesions,

which are not known to show allele loss as a non-specific feature. Thirdly, allele loss on 16p13.3 is not a common finding either as a primary or secondary phenomenon in malignant tumours.

Allele loss in benign lesions from patients with TSC suggests that the product of the TSC gene functions as a tumour or growth suppressor, in accordance with Knudsen's two hit hypothesis<sup>11</sup>. A germline alteration in the tumour suppressor gene, inherited from an affected parent, is complemented by a second somatic alteration in the allele inherited from the unaffected parent. In the one case of allele loss where we were able to study segregation of the marker, we showed that the allele of KG8 which was deleted in an angiomyolipoma was the same allele that was inherited by the patient's unaffected daughter. This would be consistent with Knudsen's hypothesis.

Although allele loss has been well described in a wide variety of malignant tumours, including breast and lung carcinomas<sup>12</sup>, as far as we know allele loss in hamartomas has not been reported previously. If we are correct in suggesting that the two hit hypothesis of Knudsen applies to the development of hamartomas in TSC, then there are several implications that need to be explored. For example, the angiomyolipomas, rhabdomyomas, cortical tubers and giant cell astrocytomas in TSC would have to be predominantly clonal in origin, which would be surprising for lesions which are a mixture of tissue types. Another prediction would be that both hits could occur as somatic events, so that similar lesions could develop unrelated to TSC, but the onset would be later and multiple lesions

would be very rare. This model would fit the data for sporadic angiomyolipomas, which are found in an older age group than angiomyolipomas associated with TSC, and are typically single lesions<sup>13</sup>. TSC accounts for approximately 60% of all cardiac rhabdomyomas<sup>14</sup>, but there is no clear data establishing any clinical differences between TSC-associated and sporadic cardiac rhabdomyomas<sup>15</sup>. Sporadic cortical tubers and giant cell astrocytomas appear to be extremely rare, which is difficult to explain on the basis of this model. Finally, does the two hit model apply to other lesions in TSC including the characteristic lesions on the skin? If so, we would again expect that similar lesions could occur in isolation. Hypopigmented macules do occur in 0.8% of healthy newborns<sup>16</sup> and shagreen patches have been reported in otherwise normal subjects<sup>17</sup>. However, the most characteristic lesions in TSC, including subependymal nodules, retinal astrocytomas, facial angiofibromas, forehead plaques and unguis fibromas are claimed to be pathognomonic of TSC and rarely if ever occur in isolation<sup>1</sup>. This also would be difficult to explain on the basis of the two hit model.

Based on evidence of allele loss in the vicinity of the TSC locus on 16p13.3 in hamartomas from TSC patients, we suggest that the TSC gene on 16p13.3 acts as a 'growth suppressor gene' analogous to the two hit model of the traditional tumour suppressor gene.

### Methodology

**Patients.** All patients were diagnosed as TSC according to the revised Gomez criteria<sup>1</sup>. The diagnosis of hamartoma or tumour was made by an independent pathologist. Normal DNA was obtained from peripheral leukocytes or from histologically normal tissue. DNA was extracted from 9 lesions found in 8 patients. For 6 of the 9 lesions, 4 renal angiomyolipomas, one cortical tuber and one cardiac rhabdomyoma paraffin embedded tissue was the only source of DNA. Polymorphic dinucleotide repeats were the only markers which could be studied in these cases. Frozen tissue was used as the source of DNA in the other three lesions (two renal angiomyolipomas and one giant cell astrocytoma). Peripheral blood was used as a source of normal DNA in six cases; paraffin embedded spleen was used as a post mortem source of DNA for the patient with the cardiac rhabdomyoma and cortical tuber; normal renal tissue adjacent to the angiomyolipoma was used as a source of normal DNA in the case of

**Table 1 Results of typing tissue samples with chromosome 16 markers**

| Tumour | D16S423 | D16S94 | D16S283 | D16S291 | KG8 | D16S259 | D16S309 | D16S85 |
|--------|---------|--------|---------|---------|-----|---------|---------|--------|
| GCA1   | +       | +      | •       | +       | •   | •       | ★       | ★      |
| AML1   | +       | +      | +       | +       | •   | •       | +       | +      |
| AML2   | -       | +      | •       | •       | •   | •       | •       | ★      |
| AML3   | +       | +      | •       | +       | ★   |         |         |        |
| AML4   | +       | +      | •       | •       | ★   |         |         |        |
| AML5   | +       | •      | +       | +       | •   |         |         |        |
| AML6   | +       | •      | •       | +       | •   |         |         |        |
| CRM1   | +       | +      | +       | +       | ★   |         |         |        |
| TUB1   | -       | -      | -       | +       | ★   |         |         |        |

Markers distal to KG8 could not be typed from paraffin embedded samples AML3-6, CRM1 and TUB1.

GCA, Giant cell astrocytoma.

AML, Renal angiomyolipoma.

CRM, Cardiac rhabdomyoma.

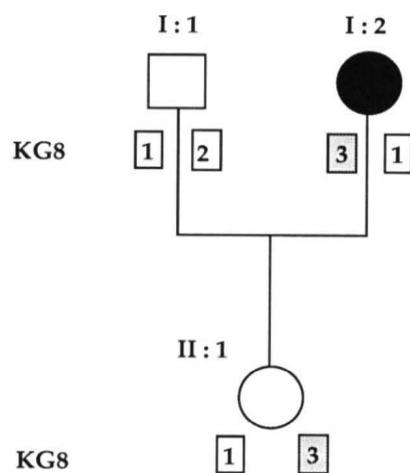
TUB, Cortical tuber.

+, No loss of heterozygosity.

•, Non-informative.

★, Loss of heterozygosity.

-, Not typed.



**Fig. 2** Pedigree of the patient with angiomyolipoma 4, showing marker data. The angiomyolipoma is deleted for allele 3. This is the same allele that the patient has passed on to her unaffected daughter (who has inherited allele 1 from her healthy father). **3**, Allele deleted in renal angiomyolipoma from I:2.



angiomyolipoma 1. Cytogenetic studies were not performed on any tissue samples. For the patient with angiomyolipoma 4, DNA was obtained from mouthwash samples from her husband and unaffected daughter.

**DNA preparation.** Leukocyte DNA and DNA from frozen tissue was extracted with phenol and chloroform according to standard methods<sup>18</sup>. DNA was extracted from archival paraffin embedded tissue using a minor modification of the method of Smith *et al.*<sup>12</sup>. Proteinase K digestion of sections was carried out in the buffer described, but for 5 days at 40 °C. The reaction was incubated at 99 °C for 10 min and then cooled. Aliquots of 1–5 µl were used as templates for a PCR reaction.

**Probes.** DNA digestion, Southern blotting, probe labelling and hybridization were performed according to standard methods<sup>18</sup>. From centromere to telomere, the markers studied on chromosome 16p13 were the CA dinucleotide repeats *D16S423* (AFM249yc5), *D16S94* (VK5AC), *D16S283* (SM7), *D16S291* (CKLH9) and KG8, an RFLP *D16S259* (GGG1) and the minisatellites *D16S309* (MS205) and *D16S85* (3'HVR). Three chromosome 9q34 dinucleotide repeat markers were used: from centromere to telomere; *ASS*, *D9S64* and *D9S66*. Other microsatellite markers typed as controls were *D3S1038* on chromosome 3p, *TH* on 11p, *D11S35* on 11q and *PAH* and *D12S56* on 12q.

PCR's were performed in 25 µl reactions containing a final concentration of 50mM KCl, 10mM Tris pH 8.0, 0.1 mg ml<sup>-1</sup> gelatin,

0.45% NP40 and 0.45% Tween 20. MgCl<sub>2</sub> concentrations (0.5mM to 2.5 mM) were used according to optimized PCR conditions. PCR's were performed either with an end-labelled primer, and analysed on a 6% polyacrylamide sequencing gel with subsequent autoradiography, or performed without radiolabelling and analysed on a 10% 10cm × 10cm polyacrylamide gel and visualised by ethidium bromide staining.

**Loss of heterozygosity.** When dinucleotide repeat markers were used, loss of an allele was considered only when no second allele could be amplified from the lesion studied, where the normal DNA showed two alleles. When VNTR's were used, allele loss was considered when there was a major imbalance between the two alleles seen in normal DNA and DNA from the affected tissue.

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