

Brief Report

MOSAICISM IN TUBEROUS SCLEROSIS AS A POTENTIAL CAUSE OF THE FAILURE OF MOLECULAR DIAGNOSIS

JOLANTA KWIATKOWSKA, PH.D.,
JADWIGA WIGOWSKA-SOWINSKA, M.D.,
DOBRAWA NAPIERALA, M.S., RYSZARD SLOMSKI, PH.D.,
AND DAVID J. KWIATKOWSKI, M.D., PH.D.

MOSAICISM is the phenomenon in which a fraction of, rather than all, germ-line and somatic cells contain a mutation or chromosomal abnormality. It occurs in all genetic disorders in which spontaneous mutations occur and has important clinical consequences for the assessment of patients with localized expression of multisystem disorders, for genetic counseling, and for molecular diagnostic testing.^{1,2}

Tuberous sclerosis is an autosomal dominant disorder characterized by the development of unusual tumor-like growths (hamartomas) in multiple organs.^{3,4} Arguably the most important hamartomas are cerebral cortical tubers, which are regions of abnormal cortical architecture with distinctive large neuronal cells. Cortical tubers cause some of the most important clinical manifestations of tuberous sclerosis: epilepsy, mental retardation, and abnormal behavior including autism.^{3,5,6} Other hamartomatous lesions in tuberous sclerosis include subependymal nodules, facial angiofibromas, subungual fibromas, forehead plaques, shagreen patches, cardiac rhabdomyomas, and renal angiomyolipomas and cysts. The incidence of tuberous sclerosis is 1 in 6000 births, and about two thirds of cases are sporadic, occurring in the absence of a family history of the disorder.^{7,8} Mutations in one of two genes, *TSC1* and *TSC2*, cause tuberous sclerosis.^{9,10}

We describe a patient with severe tuberous sclerosis in whom a mutated *TSC1* allele was present in only one third of leukocytes and in different proportions in other tissues. This case report illustrates the importance of considering mosaicism in many clinical

settings and the limitations of molecular diagnostic methods, including complete gene sequencing, for the detection of mosaicism.

CASE REPORT

An infant girl weighed 3000 g when born at term. Her mother was 20 years old, and her father was 24 years old; both were healthy. The baby's development was normal until the age of 18 months, when myoclonic seizures occurred, which were treated with corticotropin. Although the seizures stopped, the child subsequently learned few additional words and withdrew from interaction with her parents and others. At the age of 12 years she began to have absence seizures, and she was seen at a tertiary center to assess whether a genetic disease was present. Evaluation revealed a normal body habitus but limited activity and diminished social interaction. The child spoke simple sentences and could not read or count, but she could obey simple commands. On formal testing, the IQ score was less than 40. Several angiofibromas were present in the malar regions. Magnetic resonance imaging and computed tomography of the brain showed several calcified subependymal nodules, two large cortical tubers — one of which was calcified — and many smaller cortical tubers (Fig. 1). The results of renal ultrasonography, echocardiography, and retinal examination were all normal.

METHODS

Leukocyte DNA from the patient was included in a set of DNA samples from 161 patients who met formal diagnostic criteria for tuberous sclerosis.⁴ These samples were screened for mutations in the *TSC1* gene by heteroduplex analysis of all coding exons after amplification with the polymerase chain reaction (PCR).^{9,11} All patients or their parents gave informed consent, and the study was approved by the human research committee of Brigham and Women's Hospital.

Heteroduplex analysis is a method for the detection of variations in DNA sequences that depends on the different patterns of migration during electrophoresis of DNA duplexes that are mismatched at one or more internal nucleotides (Fig. 2). We used conformation-sensitive gel electrophoresis¹¹ followed by SYBR Green I or silver staining to identify heteroduplexes. Sequencing was performed with *Taq* polymerase in cycle sequencing with ³²P-labeled oligonucleotide primers. The PCR products were subcloned into pUC18. Multiple pairs of oligonucleotide primers were used to detect the wild-type allele and the mutant allele with a deletion of the adenine (A) and cytosine (C) residues at position 2122 (2122delAC) in hybridization.

The fraction of heteroduplexes formed was quantified by digital gel scanning with a Fluorimager and analysis with ImageQuant (Molecular Dynamics). The fraction of heteroduplexes was converted to the fraction of mutant alleles per cell in the DNA according to the following formula: $m = 1 - \sqrt{1 - 2 \times h}$, where m is the degree of mosaicism and h is the heteroduplex fraction. This equation reflects the fact that more mutant alleles form heteroduplexes as the fraction of mutant alleles decreases.

RESULTS

Amplification of exon 15 of *TSC1* of the patient's leukocyte DNA demonstrated a heteroduplex shift (Fig. 2) suggestive of a mutation, but the intensity of the heteroduplex bands was less than that in DNA samples from other patients, including a previously described patient with tuberous sclerosis with an identical heteroduplex shift⁹ (Fig. 3A). Sequence analysis of the patient's amplified exon 15 initially demonstrated no changes, but closer inspection revealed weak additional bands, suggestive of the pres-

From the Division of Experimental Medicine and Medical Oncology, Brigham and Women's Hospital, Boston (J.K., D.J.K.); the Institute of Human Genetics, Polish Academy of Science, Poznan, Poland (J.K., D.N., R.S.); and the Department of Developmental Neurology, University of Medical Sciences, Poznan, Poland (J.W.-S.). Address reprint requests to Dr. Kwiatkowski at the Division of Experimental Medicine, 221 Longwood Ave., Boston, MA 02115, or at kwiatkowski@calvin.harvard.edu.

©1999, Massachusetts Medical Society.

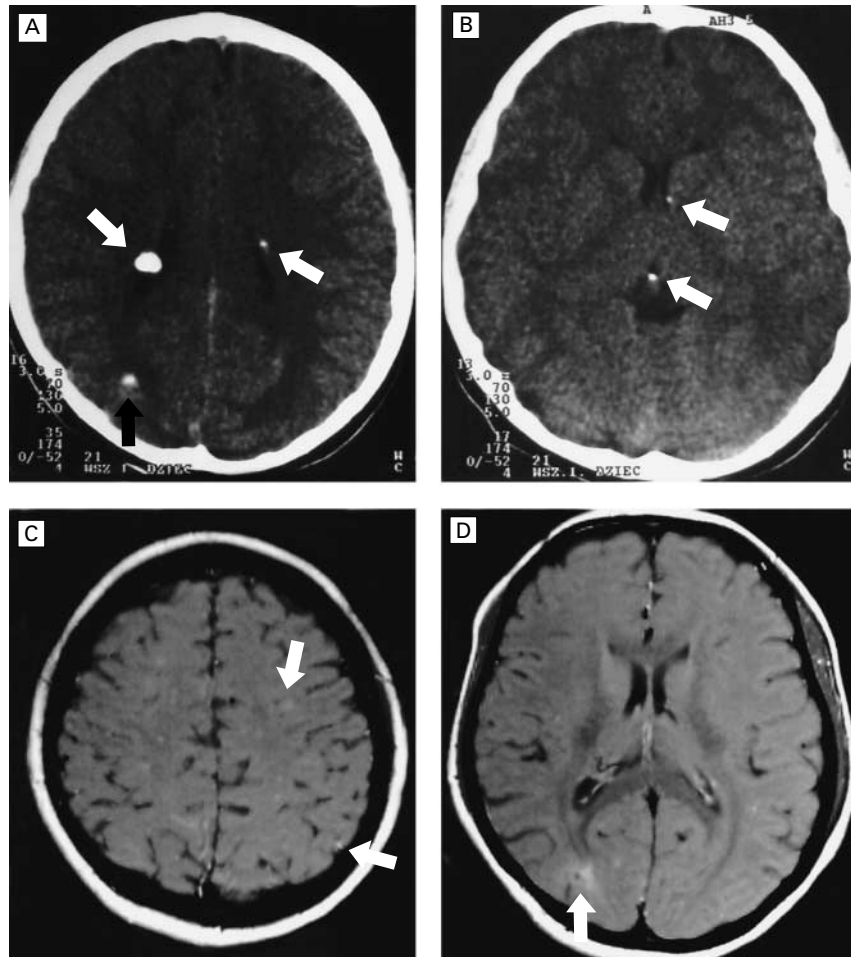


Figure 1. Computed Tomographic and Magnetic Resonance Imaging Scans of the Brain in a Patient with Tuberous Sclerosis.

In Panels A and B, computed tomographic scans show four calcified subependymal nodules (white arrows) and a calcified tuber (black arrow). In Panels C and D, magnetic resonance imaging scans show multiple tubers (arrows).

ence of mosaicism, with a reduced contribution of the mutant allele (Fig. 3B). The mutation appeared to be 2122delAC, identical to that found in the other patient with the same but more intensely staining heteroduplex shift.

To confirm this impression, we cloned the amplified product of exon 15 into a plasmid vector and analyzed the clones by hybridization. Two of 49 clones (4 percent) hybridized to a mutant-specific oligonucleotide, and sequencing confirmed that they contained the 2122delAC mutation (Fig. 3B). This deletion changes the TSC1 protein sequence after amino acid residue 634 and truncates it at residue 685; in contrast, the normal TSC1 protein has 1164 residues. This mutation is therefore an inactivating one, like other *TSC1* mutations.⁹ No other abnor-

malty was found during sequence analysis of multiple clones.

To explore the extent of mosaicism in different tissues of this patient, we amplified exon 15 in DNA prepared from urine, hair roots, and buccal mucosa (Fig. 3C). DNA from urine and hair roots formed heteroduplexes, indicating that they contained the mutant allele. However, a sample of buccal-mucosa DNA had no heteroduplex product, suggesting that the mutant allele was absent in that sample. As expected, corresponding samples from the patient's father and mother showed no heteroduplex formation.

To confirm and quantify these findings, we used PCR to amplify the region with the mutation with eight different pairs of flanking primers, and we analyzed both the original set of DNA samples and a

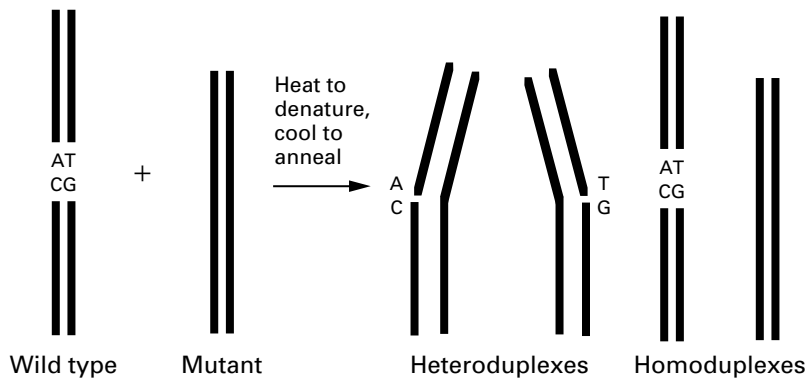


Figure 2. Heteroduplex Analysis of Amplified DNA Fragments.

PCR products consist of perfectly matching strands of complementary DNA that reflect the sequence of a person's DNA. When there is a sequence difference between the two alleles in a person's DNA — in this example, which was identified in our patient, A and C are deleted in one allele — the complementary strands can hybridize to each other, yielding two different heteroduplex molecules. The absence of the two bases in one strand causes the two unpaired bases to flip out of the helix, leading to kinking at that point and a different pattern of migration during electrophoresis.

second set. We then assessed the extent of heteroduplex formation in a quantitative manner (a representative gel is shown in Fig. 3D). The DNA sample from the patient with the same mutation as the index patient but without mosaicism had a mean (\pm SD) heteroduplex fraction of 0.51 ± 0.04 (eight samples), as compared with a predicted value of 0.50. The heteroduplex fraction in leukocyte DNA from the index patient was 0.29 ± 0.04 (eight samples), which corresponds to a frequency of the mutated allele of 0.35. DNA samples from other sites had heteroduplex fractions ranging from 0.11 to 0.33, corresponding to a frequency of the mutated allele ranging from 0.12 to 0.42.

DISCUSSION

Our patient had severe tuberous sclerosis characterized by seizures, mental retardation, and facial angiofibromas (the Vogt triad³), as well as by abnormalities on magnetic resonance imaging of the brain, and an inactivating mutation in the *TSC1* gene. However, the mutation was present in only about one third of leukocytes. Its frequency in uroepithelial DNA and hair-root DNA varied, and it was undetectable in one sample of buccal-mucosa DNA. These observations have implications with respect to both the pathogenesis of tuberous sclerosis and genetic diagnosis and counseling for this and other genetic disorders in which mosaicism occurs.

Tuberous sclerosis belongs to the family of tumor-suppressor-gene syndromes. Affected patients typically have one inactivating mutation in either the *TSC1* or *TSC2* gene in all germ-line and somatic cells. Many renal angiomyolipomas in these patients have a loss of heterozygosity (allelic loss) in either

the *TSC1* or the *TSC2* genomic region, consistent with the occurrence of a “second hit.”¹²⁻¹⁴ A lower frequency of allelic loss has also been demonstrated in other hamartomas in these patients, but this difference may reflect the mechanism by which alleles are inactivated.¹⁵ In our patient, all four types of tissue that were examined showed mosaicism, suggesting that mosaicism is present in all organs and tissues. Since this patient had cortical tubers and facial angiofibromas typical of tuberous sclerosis, our findings suggest that a hamartomatous lesion can occur if even a small proportion of cells in an organ have a *TSC1* mutation. This observation is consistent with the two-hit hypothesis for the development of hamartoma in patients with tuberous sclerosis.

The clinical presentation of tuberous sclerosis is highly variable, but the disorder is usually considered fully penetrant.^{3,4,16} Segmental expression of the phenotype is known to occur, with manifestations limited to the brain, kidney, or one side of the face, but the *TSC1* and *TSC2* mutations in such cases have not been characterized.¹⁷⁻¹⁹ There have also been several reports of patients with mosaicism for *TSC2* mutations,²⁰⁻²² but these patients have had milder clinical findings than the patient described here or have had tuberous sclerosis and polycystic kidney disease.²¹

Our case report points out a serious potential problem in the genetic diagnosis of tuberous sclerosis and other disorders caused by spontaneous mutations. Since two thirds of the cases of tuberous sclerosis are sporadic, a substantial fraction could involve mosaicism. Our results demonstrate that it is possible for a *TSC1* mutation to be present at low frequency in leukocyte DNA, despite the presence of severe dis-

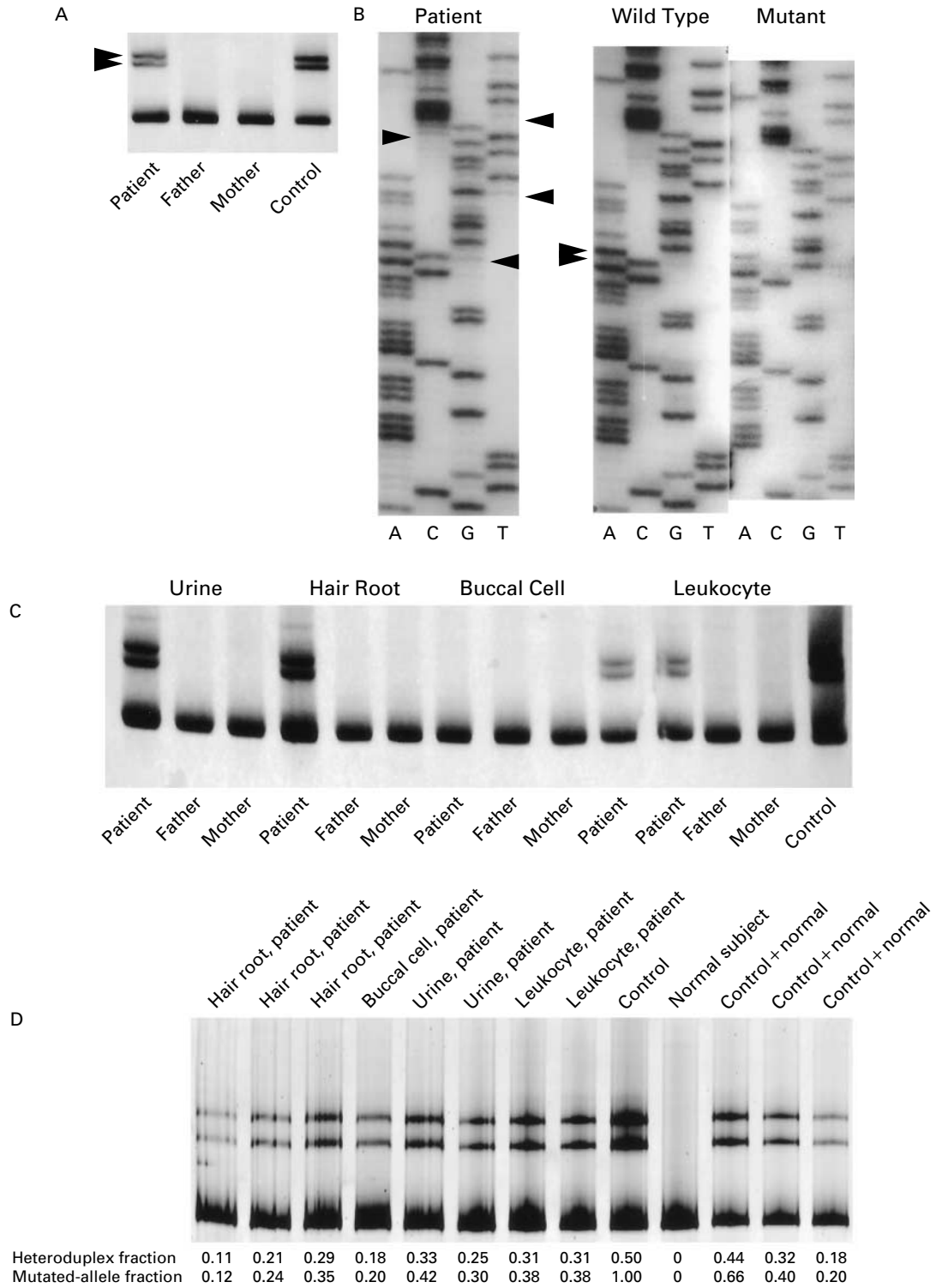


Figure 3. Demonstration of the 2122delAC Mutation and Mosaicism in the Patient.

In Panel A, heteroduplex analysis of amplified exon 15 of leukocyte DNA from the patient, her father and mother, and a control patient with tuberous sclerosis and the same mutation but no mosaicism shows heteroduplex bands in the samples from both patients (arrowheads). The staining of the heteroduplex bands from the case patient is less intense than that of the bands from the control patient. In Panel B, sequence analysis of amplified exon 15 of leukocyte DNA from the patient shows weak additional bands shifted down by 2 bp (arrowheads). The corresponding wild-type sequence, with the bases that were deleted in the mutant sequence indicated by arrowheads, and that of the mutant allele after cloning are also shown. Panel C shows the results of heteroduplex analysis of PCR products of urine, hair root, buccal mucosa, and leukocyte DNA from the patient, her father and mother, and the same control patient as shown in Panel A. No heteroduplexes are seen in the buccal-mucosa DNA product. Panel D shows the heteroduplex products derived from several sites, quantified according to the fraction of heteroduplexes and the fraction of mutated alleles (mosaicism). The buccal-mucosa samples differ in Panels C and D. The lanes in Panel D labeled "Control+normal" refer to admixtures of specimens from the patient with the same mutation but without mosaicism and a normal subject.

ease. Our method of screening for mutations was a relatively sensitive method for the detection of mutations in the presence of mosaicism. Because of the low frequency of the mutant allele, our patient's mutation probably would not have been detected by direct sequencing of the exons of *TSC1* — a method that is often considered the gold standard for the detection of mutations.²³ Moreover, the extent of somatic mosaicism in different tissues may vary to such a degree that no mutant alleles are present in leukocytes, whereas they are present in moderate amounts in other organ systems affected by tuberous sclerosis, particularly the brain. The failure to detect mosaicism could help explain why relatively few *TSC1* and *TSC2* mutations have been identified to date in patients with tuberous sclerosis,^{9,11,24-26} despite moderately intensive screening efforts.

Supported by grants from the National Institutes of Health and the National Tuberous Sclerosis Association.

We are indebted to Fiona Hall for technical assistance.

REFERENCES

- Bernards A, Gusella JF. The importance of genetic mosaicism in human disease. *N Engl J Med* 1994;331:1447-9.
- Hall JG. Somatic mosaicism: observations related to clinical genetics. *Am J Hum Genet* 1988;43:355-63.
- Gomez M. Tuberous sclerosis. 2nd ed. New York: Raven Press, 1988.
- Roach ES, Smith M, Huttenlocher P, Bhat M, Alcorn D, Hawley L. Diagnostic criteria: tuberous sclerosis complex: report of the Diagnostic Criteria Committee of the National Tuberous Sclerosis Association. *J Child Neurol* 1992;7:221-4.
- Hunt A, Shepherd C. A prevalence study of autism in tuberous sclerosis. *J Autism Dev Disord* 1993;23:323-39.
- Smalley SL, Tanguay PE, Smith M, Gutierrez G. Autism and tuberous sclerosis. *J Autism Dev Disorder* 1992;22:339-55.
- Sampson JR, Scahill SJ, Stephenson JB, Mann L, Connor JM. Genetic aspects of tuberous sclerosis in the west of Scotland. *J Med Genet* 1989;26:28-31.
- Osborne JP, Fryer A, Webb D. Epidemiology of tuberous sclerosis. *Ann N Y Acad Sci* 1991;615:125-7.
- van Slegtenhorst M, de Hoogt R, Hermans C, et al. Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. *Science* 1997;277:805-8.
- The European Chromosome 16 Tuberous Sclerosis Consortium. Identification and characterization of the tuberous sclerosis gene on chromosome 16. *Cell* 1993;75:1305-15.
- Kwiatkowska J, Jozwiak S, Hall F, et al. Comprehensive mutation analysis of the TSC1 gene: observations on frequency of mutation, associated features, and nonpenetrance. *Ann Hum Genet* 1998;62:277-85.
- Green AJ, Smith M, Yates JR. Loss of heterozygosity on chromosome 16p13.3 in hamartomas from tuberous sclerosis patients. *Nat Genet* 1994;6:193-6.
- Carbonara C, Longa L, Grosso E, et al. Apparent preferential loss of heterozygosity at TSC2 over TSC1 chromosomal region in tuberous sclerosis hamartomas. *Genes Chromosomes Cancer* 1996;15:18-25.
- Henske EP, Scheithauer BW, Short MP, et al. Allelic loss is frequent in tuberous sclerosis kidney lesions but rare in brain lesions. *Am J Hum Genet* 1996;59:400-6.
- Henske EP, Wessner LL, Golden J, et al. Loss of tuberin in both subependymal giant cell astrocytomas and angiomyolipomas supports a two-hit model for the pathogenesis of tuberous sclerosis tumors. *Am J Pathol* 1997;151:1639-47.
- Kwiatkowski DJ, Short MP. Tuberous sclerosis. *Arch Dermatol* 1994;130:348-54.
- Kerr LA, Blute ML, Ryu JH, Swensen SJ, Malek RS. Renal angiomyolipoma in association with pulmonary lymphangioleiomyomatosis: forme fruste of tuberous sclerosis? *Urology* 1993;41:440-4.
- Raymond AA, Fish DR, Stevens JM, Cook MJ, Sisodiya SM, Shorvon SD. Association of hippocampal sclerosis with cortical dysgenesis in patients with epilepsy. *Neurology* 1994;44:1841-5.
- Anliker MD, Dummer R, Burg G. Unilateral agminated angiofibromas: a segmental expression of tuberous sclerosis? *Dermatology* 1997;195:176-8.
- Verhoef S, Vrtel R, van Essen T, et al. Somatic mosaicism and clinical variation in tuberous sclerosis complex. *Lancet* 1995;345:202.
- Sampson JR, Maheshwar MM, Aspinwall R, et al. Renal cystic disease in tuberous sclerosis: role of the polycystic kidney disease 1 gene. *Am J Hum Genet* 1997;61:843-51.
- Yates JRW, van Bakel I, Sepp T, et al. Female germline mosaicism in tuberous sclerosis confirmed by molecular genetic analysis. *Hum Mol Genet* 1997;6:2265-9.
- Eng C, Vijg J. Genetic testing: the problems and the promise. *Nat Biotechnol* 1997;15:422-6.
- Jones AC, Daniells CE, Snell RG, et al. Molecular genetic and phenotypic analysis reveals differences between TSC1 and TSC2 associated familial and sporadic tuberous sclerosis. *Hum Mol Genet* 1997;6:2155-61.
- Au K-S, Rodriguez JA, Finch JL, et al. Germ-line mutational analysis of the TSC2 gene in 90 tuberous-sclerosis patients. *Am J Hum Genet* 1998;62:286-94.
- Vrtel R, Verhoef S, Bouman K, et al. Identification of a nonsense mutation at the 5' end of the TSC2 gene in a family with a presumptive diagnosis of tuberous sclerosis complex. *J Med Genet* 1996;33:47-51.