Hum Genet (2000) 106:663–668
Digital Object Identifier (DOI) 10.1007/s004390000316

ORIGINAL INVESTIGATION

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Application and evaluation of denaturing HPLC for molecular genetic analysis in tuberous sclerosis

Received: 22 February 2000 / Accepted: 21 April 2000 / Published online: 30 May 2000

Abstract Tuberous sclerosis (*TSC*) is an autosomal dominant disorder characterised by the development of hamartomas in multiple tissues and organs. *TSC* exhibits locus heterogeneity with genes at 9q34 (*TSC1*) and 16p13.3 (*TSC2*) that have 21 and 41 coding exons, respectively. The mutational spectrum at both loci is wide and previous studies have shown that 60%–70% of cases are sporadic and represent new mutations. We have formatted denaturing high performance liquid chromatography (DHPLC) for rapid screening of all coding exons of *TSC1* and *TSC2*. DHPLC analysis detected likely disease-causing mutations in 103 of 150 unrelated cases (68%), compared with 92/150 (61%) and 87/150 (58%) for single-strand conformation polymorphism analysis (SSCP) and conventional heteroduplex analysis (HA), respectively. Capital, consumable and labour costs were determined for each exon screening procedure. Estimated costs per patient sample depended on throughput, particularly for DHPLC, where a high proportion of costs are fixed, and were £257, £216 and £242 for DHPLC, SSCP and HA, respectively, assuming a throughput of 252 samples per year, or £354, £233 and £259, assuming a throughput of 126 samples per year. DHPLC had the advantages of increased sensitivity and reduced labour costs when compared with more traditional approaches to exon screening but, unless expensive DHPLC equipment is being efficiently utilised for a very high proportion of the time available, overall costs are slightly higher.

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Introduction

Tuberous sclerosis (*TSC*, MIM 191090 and MIM 191100) is an autosomal dominant multisystem disorder with an estimated prevalence of at least 1 in 10,000 live births (Osborne et al. 1991). It is characterised by the development of hamartomatous growths in multiple organs and tissues, most notably the brain, kidneys and skin (Gomez et al. 1999). Seizures, intellectual handicap and behavioural abnormalities are common in *TSC*; however, phenotypic severity is variable, both between and within families (Gomez 1991). Approximately two thirds of *TSC* cases are sporadic and represent new mutations (Sampson et al. 1989; Osborne et al. 1991).

TSC exhibits locus heterogeneity with approximately equal proportions of familial cases showing segregation with loci on chromosome 9q34 (*TSC1*) or 16p13.3 (*TSC2)*;(Povey et al. 1994). Positional cloning has identified both genes. *TSC1* has 21 exons encoding an ~8.6 kb transcript (*TSC1* Consortium 1997) and *TSC2* has 41 coding exons encoding an ~5.5 kb transcript (European Chromosome 16 Tuberous Sclerosis Consortium 1993). Loss of heterozygosity has been demonstrated at 9q34 and 16p13.3 in both *TSC*-associated and sporadic hamartomas (Green et al. 1993; Carbonara et al. 1994; Henske et al. 1996), consistent with *TSC1* and *TSC2* functioning as tumour suppressors.

Although penetrance in *TSC* is complete, or virtually so, phenotypic variability can make the determination of disease status difficult in family members of affected individuals. Normal genetic status is extremely likely if clinical and ophthalmological examination, kidney ultrasound and brain computerised tomography or magnetic resonance imaging scans are normal; however, the detection of phenotypic variants of uncertain significance is relatively common during clinical and radiographic assessment. The identification of *TSC1* and *TSC2* has intro-

duced the possibility of highly specific molecular genetic diagnosis, including early prenatal diagnosis for *TSC*.

We have previously reported comprehensive mutational analysis of both *TSC1* and *TSC2* in a cohort of 150 unrelated *TSC* patients (Jones et al. 1999a) and have identified mutations in 123 cases (82%). This and other analyses of one or both genes (Wilson et al. 1996; van Bakel et al. 1997; Ali et al. 1998; Au et al. 1998; Beauchamp et al. 1998; Kwiatkowska et al. 1998; Young et al. 1998; Benit et al. 1999; Niida et al. 1999; Mayer et al. 1999; van Slegtenhorst et al. 1999; Zhang et al. 1999) indicate that the spectrum of mutations affecting *TSC1* and *TSC2* is wide, with only a small minority of mutations having been observed recurrently. Most mutations appear to be small intra-exonic changes or splice site changes, with approximately 16% of mutations being whole or multi-exon deletions or other large rearrangements that have been observed almost exclusively at the *TSC2* locus. Mutations at the *TSC2* locus account for the majority of sporadic cases (Jones et al. 1999a). The occurrence of pathogenic mutations throughout the coding sequences of both genes suggests that the efficient screening of *TSC1* and *TSC2* coding exons for sequence variants might represent a useful approach for the analysis of the genes in a diagnostic setting.

Denaturing high performance liquid chromatography (DHPLC), originally described as a tool for mutation detection by Underhill et al. (1996), resolves homo- and heteroduplex DNA molecules by using ion-pair reverse-phase HPLC under conditions of partial helix denaturation. We have previously reported DHPLC as being highly sensitive for the detection of sequence variants and have correctly identified 99 of 103 (96%) mutant samples in a blind screen of 42 different amplimers of the *TSC1*, *TSC2* and *CFTR* genes (Jones et al. 1999b). We have now formatted DHPLC for rapid screening of all coding exons of both *TSC* genes and compared it with single-strand conformation polymorphism analysis (SSCP) and conventional heteroduplex analysis (HA) for sensitivity and cost of diagnostic application.

Materials and methods

Patients and samples

DNA was extracted from peripheral blood from 150 unrelated probands fulfilling the recently revised diagnostic criteria for *TSC* (Roach et al. 1998). The epidemiological characteristics of this patient cohort, the results of exonic screening by SSCP and HA and the evaluation for large rearrangements have been described by Jones et al. (1999a). In that study, 24/150 patient samples were found to harbour whole or multi-exon deletions or other large rearrangements, and 80 different small intragenic mutations accounting for 99/150 cases were detected by SSCP and/or HA. We have now used DHPLC to screen amplimers containing each of the small intragenic mutations together with 87 control amplimers, previously classified as wild-type by SSCP and HA, in a blind study. We have also screened, by DHPLC, the entire coding region and splice junctions of both genes in 27 patient samples in which no causative mutations had been identified by SSCP or HA.

Polymerase chain reaction

Genomic polymerase chain reaction (PCR) was carried out in 50-µl reaction volumes containing 100 ng genomic DNA, 0.5 µM primers, 0.2 mM dNTP, 5 µl reaction buffer (100 mM TRIS pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin) and 1 U AmpliTaq Gold Polymerase (Cetus). PCR primers for the amplification of *TSC1* and *TSC2* exons were as previously described (Jones et al. 1999a), except those for *TSC2* exons 10, 12, 18, 37 and 38, which were redesigned for improved amplification. *TSC1* and *TSC2* amplimers were divided into those that amplified successfully at an annealing temperature of 53°C, 55°C or 57°C. Six patient samples, including a positive (known mutant) and negative (no DNA) control for each amplimer (eight reactions in total) were amplified in eight tubes on the same microtitre plate by using an oil-free primus 9600 thermocycler (MWG-Biotech). In total, six 96-well microtitre plates were required for the amplification of all coding regions of both *TSC* genes: plate 1 (*TSC1* exons 3–14), plate 2 (*TSC1* exons 15–23), plate 3 (*TSC2* exons 2, 4, 5, 8, 11, 14, 15, 16, 17, 19 and 20), plate 4 (*TSC2* exons 3, 6, 7, 13, 21, 22, 25, 27, 28, 29, 30 and 33 fragment b), plate 5 (*TSC2* exons 1, 9, 10, 12, 23, 24, 31, 33 fragment c, 37, 39, 40 and 41) and plate 6 (*TSC2* exons 18, 26, 32, 33 fragment a, 34, 35, 36 and 38). Cycling parameters were 95°C for 12 min followed by 33 cycles at 53°C (plate 3), 55°C (plates 1, 2, 4 and 5) or 57°C (plate 6) for 1 min, 72°C for 1 min and 94°C for 1 min, with a final step of 72°C for 10 min.

DHPLC procedure

DHPLC was performed on a WAVE DNA fragment analysis system (Transgenomic, Crewe, UK). To enhance heteroduplex formation, the untreated PCR product was denatured at 95°C for 5 min followed by gradual reannealing to 50°C over 45 min. Products were automatically loaded on a DNAsep column (Transgenomic) and eluted with a linear acetonitrile (ACN)(BDH Merck) gradient in a 0.1 M triethylamine acetate buffer (pH 7), at a constant flow rate of 0.9 ml/min. The start and end points of the gradient were adjusted according to the size of the PCR product. Analysis per amplified sample took 7.2–8.5 min, including column regeneration and equilibration. Samples were analysed at the melt temperature (Tm) determined by using the DHPLCMelt software (http://insertion.stanford.edu/melt.html). Where recommended by DHPLCMelt, analysis was performed at two different temperatures. Eluted DNA fragments were detected by an UV-C detector (Transgenomic). A full list of PCR primer sequences, DHPLC run temperatures, run times and ACN gradients used are available at http://www.uwcm.ac.uk/uwcm/mg/tsc_db/dhplc2.html.

Sequencing

PCR products displaying variant DHPLC melt profiles were directly sequenced by using the Sequenase PCR Product Sequencing Kit according to the manufacturer's instructions (Amersham).

Microsatellite analysis

Evidence for biological paternity and maternity in patient 106 was assessed by genotyping microsatellite markers on chromosomes 4 (D4S43), 6 (D6S250) and 15 (LS6–1, GABRB3, IR4–3R).

Cost analysis

We evaluated the cost of mutation analysis of both *TSC* genes including PCR amplification, exon screening and sequencing of variants by DHPLC, SSCP and HA in our laboratory. The direct costs measured were equipment, consumables and labour. No allowances were made for wastage or experimental failure. Labour costs were estimated, based on the gross employment costs (salary +20%) of a clinical scientist earning £17,500 ($28,000 approx.)

per annum, assuming the following time requirements: (1) that PCR amplification of all coding exons of both genes for a batch of six patients and controls (six microtitre plates) would take 3.75 h, (2) for SSCP, that the preparation of samples (aliquoting, addition of loading buffer and denaturation), preparation of gels, electrophoresis, silver staining, washing plates and interpretation of results for all six microtitre plates (six gels in total) would take 26.25 h, (3) for HA, that the preparation of samples (aliquoting, addition of loading buffer and denaturation), preparation of gels, electrophoresis, silver staining, washing plates and interpretation of results for all six microtitre plates (six gels in total) and additional checker gels for each batch of six plates screened would take 30 h, (4) for DHPLC, that the preparation of DHPLC buffers, setting up of six microtitre plates on the DHPLC machine and analysis of results would take 6 h, (5) for sequencing, that 24 manual sequencing reactions, including the preparation of gels, electrophoresis, washing plates and interpretation of results, would take 15 h. These times were estimated, based on our previous analysis of both *TSC* genes by these techniques. Equipment costs were based on present list prices and assumed a working life of 5 years. Consumable costs were spread over a hypothetical 5-year period of analysis.

According to the principles of economic appraisal, costs incurred in later years represent less of a financial burden than costs incurred earlier. To account for this, future costs need to be adjusted by using a technique known as discounting (Drummond et al. 1997). We have accordingly discounted all costs other than those in the base year by using a 6% discount rate.

Costs per patient sample screened were calculated for a throughput of 126 and 252 samples annually (a workload of three or six samples each week for 42 weeks per year) over a 5-year period. Sequencing costs per sample were based on the number of sequencing reactions (exactly 100) that were required to characterise variant elution profiles in 23 additional diagnostic samples that we completely screened by DHPLC (A. C. Jones, unpublished). The sequencing requirements following SSCP and HA were estimated to be similar to that for DHPLC, despite their lower sensitivities for detecting sequence variants, as further sequencing reactions were necessary to confirm polymorphisms in cases where clearly pathogenic mutations were not detected. Sequencing equipment costs were higher for DHPLC, because of the necessity of purchasing electrophoresis equipment (i.e. gel tank, gel drier and vacuum pump) that constituted part of the capital costs for gel-based SSCP and HA. Of the total cost per sample, 20% was allocated to overheads accounting for utilities, laboratory licences and other operating inputs, such as freight and insurance. Costs of DNA extraction, the use of additional techniques for the confirmation of mutations or polymorphisms (such as restriction digestion or cloning), confirmation of the presence of the mutation in the original DNA sample, independent review of the data, report writing and secretarial and administrative costs were not included.

Results

Sensitivities of DHPLC, SSCP and HA

Of the 80 different mutations previously detected by SSCP or HA in 99/150 unrelated cases with a clinical diagnosis of definite *TSC* (nine mutations were seen recurrently in 28 cases), 78 were correctly distinguished from 87 wild-type alleles in a blind DHPLC analysis (Fig. 1). DHPLC failed to detect two mutations in a 324-bp amplimer containing *TSC2* exon 29, Q1148X, C to T at 3460 bp (one case) and Q1192X, C to T at 3592 bp (two cases). The GC content of this fragment was high (65%), particularly in the region of these mutations. Melting of the region during DHPLC analysis may have been inadequate. Both mutations were detectable when the temperature for analysis was raised by 2°C. All 87 wild-type controls were correctly scored as normal in the blind screen.

DHPLC analysis of all coding exons of *TSC1* and *TSC2* in 27/150 unrelated cases in whom mutations had

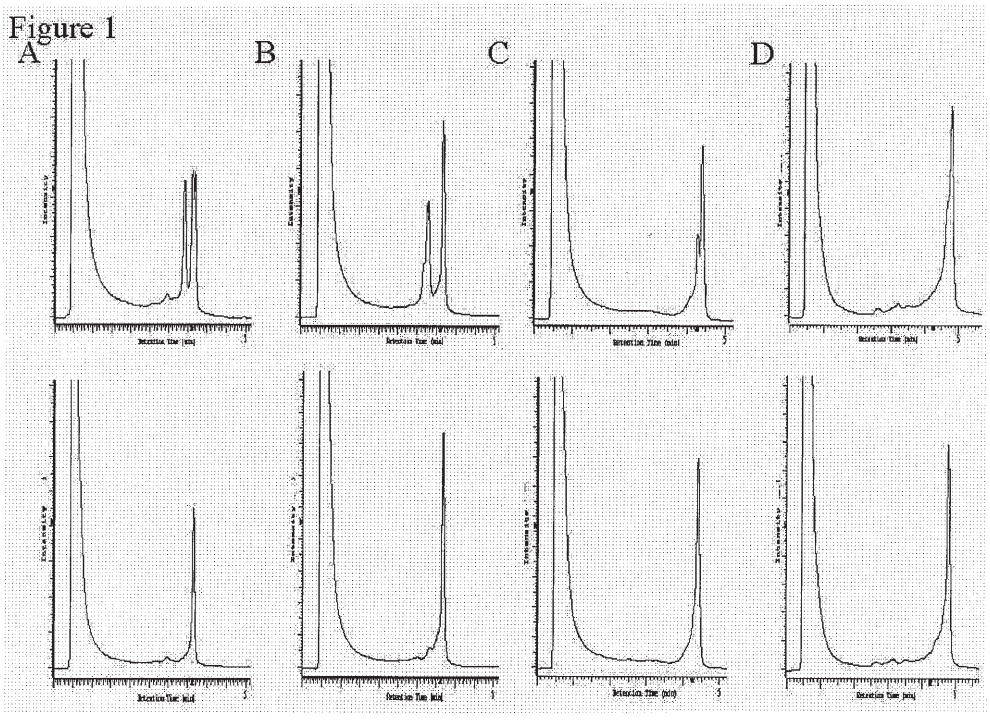

Fig. 1A–D DHPLC detection of *TSC1* and *TSC2* mutations. DHPLC chromatograms of *TSC1* and *TSC2* germline mutations. **A** The 241-bp amplimer containing *TSC1* exon 4 analysed at 59°C. *Top* 328–2 A→G. *Bottom* Wild-type. **B** The 276-bp amplimer containing *TSC1* exon 20 analysed at 60°C. *Top* S836X (C→G at 2728 bp). *Bottom* Wild-type. **C** The 244-bp amplimer containing *TSC2* exon 24 analysed at 62°C. *Top* 2855+1 G→T. *Bottom* Wild-type. **D** The 222-bp amplimer containing *TSC2* exon 19 analysed at 62°C. *Top* L733P (T→C at 2216 bp). *Bottom* Wild-type. The degree of separation of homo- and heteroduplex DHPLC profiles is variable and can result in clearly resolved mutant peaks (**A, B**) or more subtle changes (**C, D**)

Table 1 Mutations detected by DHPLC but not by SSCP or HA

Patient	Location	Mutation	Nucleotide change	Type of mutation	Previously reported
169	*TSC1* Intron 19	2724–1G→C	G to C at 2724–1bp	Splice	No
106	*TSC2* Exon 19	L733P	T to C at 2216 bp	Missense	No
013	*TSC2* Intron 20	2373+1G→A	G to T at 2373+1 bp	Splice	No
083	*TSC2* Intron 20	2374–1G→A	G to A at 2374–1bp	Splice	No
249	*TSC2* Exon 26	R1032X	C to T at 3112 bp	Nonsense	No
338	*TSC2* Intron 29	3628+1G→A	G to A at 3628+1 bp	Splice	No
120	*TSC2* Exon 34	Y1505X	C to A at 4533 bp	Nonsense	No

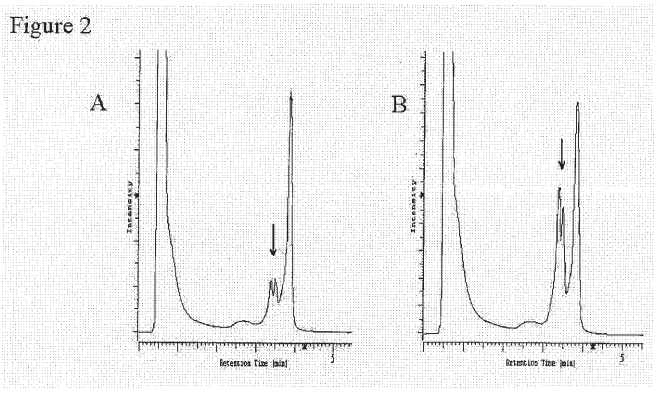

Fig. 2 DHPLC chromatograms of *TSC1* exon 20 in patient 169 and his affected father. The mutant profile (*arrow*) is reduced in the paternal sample (**A**) compared with that in his son (**B**), suggesting that the father is a mosaic for the change. Sequencing identified the splicing mutation 2724–1G→C

not been identified previously, despite screening of all coding exons by SSCP and HA and screening for whole exon and other large rearrangements, revealed variant elution profiles in 10 cases. Sequencing identified seven novel and likely disease-causing mutations (Table 1) and three unique *TSC2* intronic variants (-25G→T [5' UTR], 959–55A→T, and 4024–8C→T).

The splicing mutation 2724–1G→C in *TSC1* intron 19 was detected by DHPLC screening in patient 169. His father was also affected by *TSC*, but very mildly. DHPLC analysis also detected the mutation in a blood DNA sample from the father but the elution profile from the mutant allele was markedly reduced, indicating that he was probably a somatic mosaic for the change (Fig. 2). The previously unreported *TSC2* missense change L733P identified in patient 106, a sporadic case, was shown to have occurred de novo by analysis of parental DNA samples; the analysis of five microsatellite markers was consistent with biological parenthood.

Sensitivities of DHPLC, SSCP and HA for the detection of mutations in 150 unrelated *TSC* cases were therefore 68%, 61% and 58%, respectively (Table 2). The proportions of all cases with point mutations detected during the study that were identified by each of the techniques were 97%, 87% and 82% for DHPLC, SSCP and HA, respectively.

Table 2 Sensitivities of DHPLC, SSCP and HA for the detection of mutations in 150 unrelated *TSC* cases

Method	*TSC1*	*TSC2*	Total	Sensitivity
DHPLC	23	80	103	68%
SSCP	18	74	92	61%
HA	20	67	87	58%
Total	23	83 (107[a])	106 (130[a])	

[a]Including 24 gross rearrangements not detectable by exon scanning strategies

Cost analysis

The laboratory costs of PCR amplification and exon screening of both *TSC* genes, including the characterisation of mutations by sequencing was £257, £216 and £242 per sample for DHPLC, SSCP and HA, respectively, when screening 252 samples annually, and £354, £233 and £259 when throughput was decreased to 126 samples annually (Table 3). The total labour time required to amplify, screen and sequence variants for a batch of six samples was estimated at 24.75 h for DHPLC, 45 h for SSCP and 48.75 h for HA. Therefore, estimated labour costs per sample screened were significantly higher for SSCP (£81) and HA (£88) than for DHPLC (£44). Total consumable costs per sample screened were £91, £85 and £100 for DHPLC, SSCP and HA, respectively, when screening 252 samples annually, and £95, £86 and £101, respectively, when annual throughput was reduced to 126 samples.

Discussion

The *TSC1* and *TSC2* genes are large, of complex genomic structure and exhibit a wide mutation spectrum. We have developed a DHPLC screen for all coding regions of both *TSC* genes and evaluated the sensitivity and cost of this approach in comparison with SSCP and HA, the traditional approaches to exon screening for mutation detection in diagnostic laboratories.

In the present study of 150 unrelated patients with *TSC*, mutations were detected in 103/150 (68%) cases by DHPLC compared with 92/150 (61%) by SSCP and 87/150 (58%) by HA. Two mutations, missed by DHPLC at the Tm, were readily detectable when analysis was performed at the Tm +2°C. We have previously shown that the sensitivity of DHPLC for the detection of point mutations is marginally increased by screening at both the Tm

Table 3 Breakdown of costs for genetic testing of both *TSC* genes using DHPLC, SSCP and HA over a 5-year period

Method		SSCP (£)		HA (£)		DHPLC (£)	
	Annual throughput	126 samples	252 samples	126 samples	252 samples	126 samples	252 samples
PCR	Laboratory costs	36,468	62,591	36,468	62,591	36,120	62,049
	Labour	4241	8480	4241	8480	4241	8480
	Total	40,709	71,071	40,709	71,071	40,361	70,529
	Cost per sample	65	56	65	56	64	56
Mutation analysis	Laboratory costs	18,551	29,480	28,092	48,549	100,665	114,389
	Labour	29,684	59,367	33,926	67,851	6785	13,570
	Total	48,235	88,847	62,018	116,400	107,450	127,959
	Cost per sample	77	71	98	92	171	102
Sequencing	Laboratory costs	16,054	31,739	16,054	31,739	20,825	36,510
	Labour	17,382	34,766	17,382	34,766	17,382	34,766
	Total	33,436	66,505	33,436	66,505	38,207	71,276
	Cost per sample	53	53	53	53	61	57
Total		122,380	226,423	136,163	253,976	186,018	269,764
Overheads (20%)		24,476	45,285	27,233	50,795	37,204	53,953
Total (including overheads)	Cost per sample	146,856	271,708	163,396	304,771	223,222	323,717
Total cost per sample screened (assuming £1=$1.6)		233 ($373)	216 ($346)	259 ($414)	242 ($387)	354 ($566)	257 ($411)

and Tm +2°C (Jones et al. 1999b). However, this greatly increases the cost per sample screened, and analysis at multiple temperatures have only been performed when recommended by the DHPLCMelt software. Gross rearrangements that are not detectable by any exon screening method are found almost exclusively at the *TSC2* locus and have previously been shown to account for at least 24/150 (16%) of cases in the present series (Jones et al. 1999a). We have still not identified *TSC1* or *TSC2* mutations in 20 of 150 cases (13%) and genomic sequence analysis of the *TSC1* and *TSC2* genes is ongoing in these patients. Intronic or regulatory sequence changes and low-level mosaic mutations are likely to account for at least some of these cases. Somatic mosaicism has been reported to account for ~10% of de novo TSC mutations (Verhoef et al. 1999) and could potentially result in the failure of molecular genetic diagnosis, because of an inability to detect mutant alleles present at low frequency (Kwiatkowska et al. 1999). In this study, we have been able to detect a mutant allele that appears to be present at low frequency in DNA extracted from a peripheral blood sample. The mutant profile in this case, although much smaller than that of the wild-type, has been readily detected. However, in cases where mutations result in more subtle profile changes, mosaic alleles present at a similar frequency may be more difficult to detect.

Estimated costs per sample screened were dependent on throughput, particularly for DHPLC, where a high proportion of costs are fixed. SSCP and HA were cheaper alternatives to DHPLC, particularly for lower sample throughput. However, consumable costs were lower for DHPLC than HA, for both high and low throughput senarios. We estimated the cost per sample screened to be £257, £216 and £242 per sample for DHPLC, SSCP and HA, respectively, when screening 252 samples annually, increasing to £354, £233 and £259 when screening 126 samples annually. DHPLC was less labour-intensive, requiring an estimated 24.75 h for PCR amplification, exon screening and sequencing of variants, for six samples, compared with 45 h and 48.5 h for SSCP and HA, respectively. Sequencing costs were lower for SSCP and HA than DHPLC, which required the purchase of additional equipment. However, sequencing costs for all three methods would have been identical if performed on an automated sequencer. The true cost of screening a sample in the diagnostic setting will be significantly higher, as additional costs for wastage, experimental failures, confirmation of mutations on original DNA samples prior to reporting, independent data review and the writing and issuing of reports and secretarial and administrative costs were not included in our study.

A number of other mutation detection strategies have been employed at the *TSC1* and *TSC2* loci. The protein truncation test (PTT) has been used to screen for mutations in both *TSC1* and *TSC2* (van Bakel et al. 1997; Benit et al. 1999; Mayer et al. 1999) and its sensitivity in 48 samples from unrelated *TSC* cases has been found to be 58% (Mayer et al. 1999), which is comparable to that for SSCP and HA (Jones et al. 1999a; Niida et al. 1999). *TSC2* missense mutations are frequent in patients with *TSC* (23/150 cases or 15% in the current series) and are not detected by PTT as they fail to result in a truncated product. The relatively low transcript levels in white blood cells further limits the utility of PTT for the analysis of *TSC1* and *TSC2* mutations. All investigators using PTT for mutation detection have first established cell

lines from patients. No data regarding the cost of screening both genes by PTT has been reported.

A comprehensive screen of the coding region of *TSC1* has also been reported by means of two dimensional denaturing gradient gel electrophoresis (Dabora et al. 1998). However, the technique is labour-intensive and technically challenging, with regard to both development and implementation in the diagnostic laboratory; a similar strategy for the larger and more frequently mutated *TSC2* gene has not been reported.

The highly variable *TSC* phenotype, the concerns expressed by family members over their own genetic status, and the expense, inconvenience and sometimes non-definitive outcome of traditional clinical and radiographic investigation provide strong arguments for continuing research and development of molecular genetic diagnosis for *TSC*.

Acknowledgements We thank Dr. David Ravine, Laz Lazarou and Jenny Myring for discussion and administrative support during the course of this work. This work was supported by grants from the Welsh Scheme for the Development of Health and Social Research, the Tuberous Sclerosis Association, the National Tuberous Sclerosis Association and the Medical Research Council.

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