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ARTICLE

Comparison of fluorescent single-strand conformation polymorphism analysis and denaturing high-performance liquid chromatography for detection of *EXT1* and *EXT2* mutations in hereditary multiple exostoses

Carol Dobson-Stone[1], Roger D Cox[2], Lorne Lonie[1], Lorraine Southam[2], Maria Fraser[3], Carol Wise[4], François Bernier[5], Shirley Hodgson[6], Daniel E Porter[3], A Hamish RW Simpson[3] and Anthony P Monaco[1]

[1]The Wellcome Trust Centre for Human Genetics, and [3]Nuffield Department of Orthopaedic Surgery, University of Oxford, Oxford, UK; [2]Medical Research Council, Mammalian Genetics Unit, Harwell, UK; [4]Scottish Rite Hospital for Children, Dallas, Texas, USA; [5]Alberta Children's Hospital, Alberta, Canada; [6]Division of Medical and Molecular Genetics, Guy's Hospital, London, UK

***EXT1* and *EXT2* are two genes responsible for the majority of cases of hereditary multiple exostoses (HME), a dominantly inherited bone disorder. In order to develop an efficient screening strategy for mutations in these genes, we performed two independent blind screens of *EXT1* and *EXT2* in 34 unrelated patients with HME, using denaturing high-performance liquid chromatography (DHPLC) and fluorescent single-strand conformation polymorphism analysis (F-SSCP). The mutation likely to cause HME was found in 29 (85%) of the 34 probands: in 22 of these (76%), the mutation was in *EXT1*; seven patients (24%) had *EXT2* mutations. Nineteen of these disease mutations have not been previously reported. Of the 42 different amplicon variants identified in total in the cohort, 40 were detected by DHPLC and 39 by F-SSCP. This corresponds to mutation detection efficiencies of 95% and 93% respectively. We have also found that we can confidently distinguish between different sequence variants in the same fragment using F-SSCP but not DHPLC. In light of this, and the similarly high sensitivities of the two techniques, we propose to continue screening with F-SSCP.** *European Journal of Human Genetics* (2000) **8**, 24–32.

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Introduction

Single-strand conformation polymorphism analysis (SSCP) and denaturing high-performance liquid chromatography (DHPLC) are both techniques that can detect point mutations in a DNA fragment of interest.[1,2] They are complementary methods, in that SSCP detects base changes in single-stranded DNA (ssDNA), whereas DHPLC utilises double-stranded DNA (dsDNA). The basic techniques have been well established, but the application of four-colour fluorescence technology to visualise products on SSCP gels[3] and the incorporation of DHPLC into an integrated mutation detection system such as the Transgenomic WAVE™ DNA Fragment Analysis System[4] are both recent adaptations. These modifications have various advantages in terms of ease of application, increased throughput and, most importantly, point mutation detection efficiency.

Correspondence: Anthony P Monaco, Wellcome Trust Centre For Human Genetics, Roosevelt Drive, Headington, Oxford OX3 7BN, UK. Tel: +44 1865 287503; Fax: +44 1865 287501; E-mail: anthony.monaco@well.ox.ac.uk
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In fluorescent SSCP (F-SSCP), DNA is denatured then run under non-denaturing conditions: the separated strands are therefore free to adopt three-dimensional conformations dependent on their sequence. A single base-pair alteration can therefore cause a conformational change that will alter the electrophoretic mobility of the molecule when run on a non-denaturing polyacrylamide gel. If the DNA fragment is fluorescently labelled on both strands, sufficient variation in mobility relative to a wild-type DNA control can be detected on a fluorescent image analyser, interpreted as a sequence change and analysed accordingly.

In DHPLC, if a DNA fragment heterozygous for a sequence change is heat-denatured then slowly cooled, a mixture of homo- and heteroduplexes is formed. The heteroduplexes, due to mismatch pairing, will form weaker interactions with a hydrophobic column matrix when partially denatured and will therefore be eluted sooner than homoduplexes during reverse-phase ion-exchange HPLC. DNA fragments containing heterozygous base changes can therefore be identified by elution peak pattern variations relative to those from homozygous control DNA.

Hereditary multiple exostoses (HME, MIM no. 133700) is an autosomal, dominantly inherited disorder characterised by the presence of multiple, cartilage-capped osteochondromas (exostoses) that develop mainly from the juxtaepiphyseal regions of the long bones. The exostoses develop during childhood and grow until closure of the growth plate at puberty.[5,6,7] It is the most frequent of all skeletal dysplasias, with an estimated prevalence of 1/50 000 in the population.[8] Complications include: pressure on neighbouring nerves or blood vessels; skeletal deformity and short stature; limb length inequalities; and difficulty in joint movement and arthritis. Several operations may be required to remove the most severe growths. The gravest consequence of the disease is the possible transformation of a benign exostosis to a malignant osteo- or chondrosarcoma. The transformation rate of any single exostosis is very low, but the probability of an individual developing a malignant tumour is higher and depends on the number of exostoses present. Malignant transformation is observed in 1–5% of HME patients.[5,7,8]

Three loci, on chromosomes 8q24.1 (*EXT1*),[9] 11p11–p13 (*EXT2*)[10,11] and 19p (*EXT3*)[12] were identified through linkage studies as being involved in the development of HME. *EXT3* has not yet been cloned, but *EXT1* and *EXT2* were cloned and sequenced in 1995 and 1996 respectively.[13–15] Loss of heterozygosity found on chromosomes 8 and 11 in chondrosarcomas[16,17] and the increased relative risk conferred by HME of chondrosarcoma development indicate that *EXT1* and *EXT2* act as tumour suppressor genes. Two recent studies[18,19] have implicated the *EXT* genes in synthesis and display of cell surface heparan sulphate glycosaminoglycans (GAGs). GAGs are believed to act as cofactors of several signalling pathways affecting cell growth and differentiation,[20] consistent with a possible role in tumour growth.

Several mutations in *EXT1* and *EXT2* responsible for the HME phenotype have been published:[14,21–24] most of these are loss-of-function mutations, supporting the postulated tumour suppressor function of these gene products. Additional mutation screening of *EXT1* and *EXT2* could enable further elucidation of the function of these genes, and will facilitate a possible correlation of phenotype with genotype.

Both F-SSCP and DHPLC have been claimed to enable rapid, robust scanning of unknown mutations with high detection rates. We decided to perform a direct comparison between the two technologies in order to ascertain the better mutation screening strategy for the *EXT1* and *EXT2* genes; and to establish the strengths and weaknesses of each technique to enable their effective utilisation in future applications.

Materials and methods

Patients

Thirty-four probands were selected from unrelated families with clinical symptoms of HME. Criteria for inclusion in the study were three or more radiographically confirmed osteochondromas. Twenty-eight families were referred from the Nuffield Orthopaedic Centre, Oxford, UK. DNA samples from HME families were also provided from: the Scottish Rite Hospital for Children, Texas, USA (four families); Alberta Children's Hospital, Alberta, Canada (one family) and Guy's Hospital, London, UK (one family).

DNA extraction

Genomic DNA was extracted either from blood, using the Nucleon DNA extraction kit (Scotlab, Lanarkshire, UK), or from buccal swabs, by use of the protocol described previously.[21]

Study layout

In order to conduct the study as an objective comparison, genomic DNA was aliquoted into duplicate tubes and one set each given to two operators, one using F-SSCP and one using DHPLC for sequence variant detection. Each operator worked independently and neither had any prior knowledge about the nature or presence of mutations in the proband DNA.

PCR amplification of exons

For the proband of each family, *EXT1* and *EXT2* exons with flanking intronic regions were PCR amplified by use of specific primer pairs (Table 1). For F-SSCP, EXT1 primer pairs were ordered with 5' HEX, 6-FAM or TET fluorescent dye labels; HEX, 6-FAM or NED dyes (offering better spectral separation) were the labels on the F-SSCP EXT2 primers. The primer pairs for EXT2 exons 2–14 were designed from sequences available from Genbank (accession numbers U67356–U67368). F-SSCP cycling conditions were as follows: 94°C for 18 min; 35 cycles of 94°C for 30 s, the annealing

temperature (Table 1) for 30 s, and 72°C for 30s; 72°C for 5 min. PCR reactions were carried out on a PTC 225 Thermal Cycler (MJ Research Inc., Massachusetts, USA) using AmpliTaq Gold™ DNA polymerase (PE-Applied Biosystems, Cheshire, UK). DHPLC cycling conditions were as follows: 94°C for 2 min; a touchdown of 14 cycles (94°C for 1 min; 7.5°C above the annealing temperature with –0.5°C per cycle for 1 min; 72°C for 1 min); 25 cycles of 94°C for 1 min, the annealing temperature for 1 min, 72°C for 1 min; 72°C for 5 min. PCR amplification was carried out using the Expand™ High Fidelity PCR system (Boehringer Mannheim UK, Sussex, UK), on the thermal cycler detailed above.

F-SSCP analysis

The protocol outlined below was based on recommendations set out in the 'PCR SSCP Analysis' applications note supplied by PE-Applied Biosystems. Initially, each different DNA

Table 1 Primers used to amplify *EXT1* and *EXT2* exons[a]

Gene, exon and primer	*Primer sequence (5′ to 3′)*	*PCR product size (bp)*	*PCR conditions*[c]
EXT1, exon 1 (1A) F[b]	TGGGAAACTTGGGTGATTCTT	164	a
EXT1, exon 1 (1A) R	GGCTGTGGCTCCTCGATGC		
EXT1, exon 1 (1B) F	CTCAGCTGGCTCTTGTCTCG	235	b
EXT1, exon 1 (1B) R	GTTGGCATCTCGCTTCTGC		
EXT1, exon 1 (2) F	CCCTTCGTTCCTTGGGATC	276	a
EXT1, exon 1 (2) R	GACAAAGAGGCACGCCTG		
EXT1, exon 1 (3) F	GTTACCAAAACATTCTAGCG	268	a
EXT1, exon 1 (3) R	CTTTGGCCAGCATCGCCTGG		
EXT1, exon 1 (4) F	TTTATATTCCGGCACTTGGC	242	a
EXT1, exon 1 (4) R	TCCCTGTCAGGTACCTCTTCC		
EXT1, exon 1 (5) F	TTCAACACCATCCCTCCTC	246	a
EXT1, exon 1 (5) R	CAAGGCTGACTCCCAAAGAC		
EXT1, exon 11 F	GCACTTCTCTCATATTATCC	289	c
EXT1, exon 11 R	AAGAGAGAGCAGCTTGAC		
EXT2, exon 2 (1) F	GTCTTTTCAAGTGTCATTTGC	235	a
EXT2, exon 2 (1) R	CCAAAACTGAAACATGCCAG		
EXT2, exon 2 (2) F	AAGAATGAAGACCAAGCACC	247	b
EXT2, exon 2 (2) R	GAAGCCACAGCGATAGACAT		
EXT2, exon 2 (3) F	CAGCCGACAGTCCCATCCC	258	b
EXT2, exon 2 (3) R	CGATGGAGGGAACAAACAGA		
EXT2, exon 2 (4) F	CTTTGGCGTCTCTGTCAGCA	227	b
EXT2, exon 2 (4) R	CAAGTATCTCCTGGGGGCTG		
EXT2, exon 3 F	GACTCTTGTCTTTTCATAGTT	192	b
EXT2, exon 3 R	ATCTTGAACCCATCATAAGG		
EXT2, exon 4 F	GTAATTCCTGTTCCTCTCCAC	244	b
EXT2, exon 4 R	CACAGATTCAGTAAAGGCAC		
EXT2, exon 5 F	CTGCAATTTTCCAATCACCTG	270	b
EXT2, exon 5 R	TCCTGAGCCTTTGCGAGAG		
EXT2, exon 6 F	CTAGTTTGTAATCTCTTGCCT	220	b
EXT2, exon 6 R	CGCAGAACCACTAATGTAGA		
EXT2, exon 7 F	CTGTGAAGGGCTGTGTGTATG	200	b
EXT2, exon 7 R	CCAGTCAAGGCCACCATTTC		
EXT2, exon 8 F	GTCTCGCTTGCTCACTTAAAA	233	b
EXT2, exon 8 R	CTTCCACCCACCCTGACAG		
EXT2, exon 9 F	GCTTTTCTGACCCGTGTTAAT	270	b
EXT2, exon 9 R	CCATCCAAAATTGATCCAGC		
EXT2, exon 10 F	CTCACAAAAGTTAGGAGAAT	251	d
EXT2, exon 10 R	TATTAAACATATAAACACACT		
EXT2, exon 11 F	TGGTTGCTGTCTGAATTGGGA	242	e
EXT2, exon 11 R	GTATCATTCTCTCAGTTTTGT		
EXT2, exon 12 F	TTATCAGCTAAAGGGAACTG	227	b
EXT2, exon 12 R	CCCAAGATCACAAAGCAAGT		
EXT2, exon 13 F	AGCATGATTTTATTGTCCTTG	198	b
EXT2, exon 13 R	GGCAGGAAATAGAGATCAGA		
EXT2, exon 14 F	CTCCTCCCCACCTCCTCTC	215	f
EXT2, exon 14 R	CCCTCTGTCCCAGCCTCAC		

[a]Sequences for *EXT1* exons 2–10 are reported in the article by Wells *et al*[23]
[b]Due to the large size of *EXT1* exon 1 and *EXT2* exon 2, we used overlapping sets of primers to amplify these regions. Fragment numbers are indicated in parentheses.
[c]Key to F-SSCP PCR conditions (°C annealing temperature, mM $MgCl_2$ concentration): a – 58, 2; b – 58, 2.5; c – 54, 2.5; d – 58, 4; e – 54, 2; f – 62, 2.

fragment was amplified from an unaffected control individual and purified by use of the QIAquick PCR purification kit (QIAGEN, Surrey, UK). 1.5 μl of each fragment were concentrated by incubation of open tubes at 55°C for 10 min. These were denatured by incubation at 95°C for 3 min in the presence of 2–3 μl loading dye (80% deionised formamide, 33 mM NaOH, 10 mM EDTA and 10 mg/ml blue dextran), followed by chilling on ice. 0.5 μl of a ROX- or TAMRA-labelled custom size standard, details of which are given below, was added to each sample before loading to enable subsequent lane alignment. Each different fragment was loaded in a separate well and run under both conditions detailed below, to determine their relative electrophoretic mobilities.

Amplified DNA fragments from each proband were then pooled in combinations that allowed peaks corresponding to each amplicon to be distinguished from each other (available on request). These pools, containing up to six different amplicons, were then purified and prepared for loading as above. On each gel, one denatured and one native wild-type control pool were loaded, to respectively eliminate dsDNA peaks and run-to-run variations from the F-SSCP results.

Synthesis of F-SSCP size standard

The size standard was created by PCR amplifying from genomic DNA using one ROX- or TAMRA-labelled primer and one non-labelled primer. Using a combination of different primers, 14 singly-labelled PCR products with lengths ranging from 146–953 bp were generated. These fragments were purified as above and pooled such that the fluorescent peak intensities generated were approximately equal. The primers were designed from a region of the pterin-4a alpha-carbinolamine dehydratase gene (*PCBD*, accession no. L41560) and are as follows (5'–3' sequence):

(1) GCT AGT GAC TCC CTC CTG TTC;
(2) TCG AAC AAG TAG CAG TGT CCA;
(3) AAG CAG CCA GTG GAA GCT AA;
(4) TTC ACC CTG TAT CAC AGC TTC;
(5) AGC CTT CAG AAT GTG TCA GAG;
(6) CAC ATC ACG CTG AGC ACC CAT;
(7) CTG GAA GGC CGT GAT GCC ATC TTC;
(8) GCA GGC TGG CAA AGC ACA CAG GCT GA;
(9) GCT ACT TGT TCG ATG AAG CTG GC;
(10) CTG GAC TCC CAG TTC AGT CA;
(11) AAA TTA GTG TAA CAG AGC CC;
(12) TTT GTA AGG TGA CCC CAT CAG;
(13) GGT CTA AAT TCC TGG TGT TG;
(14) TTC ACC CTG GAT CAC AGC TTC;
(15) ACT CCT CTA TAA TCA TTT CCC C;
(16) TGT GGA CCT TGT TGT ACA CGT;
(17) AGC CTT CAG AAT GTG TCA GAG.

Primers 1–5 only were fluorescently labelled; primer combinations used were as follows (forward primer first): 1&9; 1&10; 2&11; 3&12; 1&13; 1&11; 2&14; 6&4; 1&14; 3&15; 7&5; 8&5; 3&16; 3&17. Fragments were PCR amplified with the following cycling conditions: 94°C for 15 min; 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 3 min; 72°C for 10 min. The TAMRA-labelled size standard was used with EXT1 fragment pools, while the ROX-labelled version was used for EXT2 pool lane alignment.

F-SSCP running conditions

Samples were loaded on a 6.5% 37.5:1 acrylamide gel containing 1 × TBE buffer and 5% (w/v) glycerol. Electrophoresis was performed on an ABI PRISM 377 DNA Sequencer (PE-Applied Biosystems) with an attached NESLAB RTE-101 water bath (NESLAB Instruments Inc., New Hampshire, USA) to maintain accurate temperature control during electrophoresis. Runs were carried out for 8 h at 60 W, 60 mA, 4000 V at 18°C in 1 × TBE buffer. Duplicate runs were performed at 25°C with gels containing 10% glycerol. Gel lanes were aligned and analysed using the GeneScan Fragment Analysis program (PE-Applied Biosystems).

DHPLC analysis

DHPLC analysis was carried out using the WAVE™ DNA Fragment Analysis System (Transgenomic, Cheshire, UK). Crude PCR products, which had been subjected to denaturation at 95°C for 4 min, followed by gradual reannealing from 95°C to 25°C over 35 min, were injected into a DNASep^{R} column. The column mobile phase consisted of a linear acetonitrile gradient in a 0.1 M triethylamine acetate buffer (TEAA), achieved by mixing of buffers A (0.1 M TEAA), and B (25% acetonitrile in 0.1 M TEAA). The calculated gradient at a flow rate of 0.9 ml/min was run for all the amplicons at the relevant column temperature(s) for each fragment (details on request).

DHPLC gradient and temperature optimisation

For each different amplicon, the wild-type control was initially applied and subjected to a 16 min universal gradient from 40% to 72% buffer B at 50°C. From this, a 5 min gradient containing the elution peak was selected to be used in subsequent runs. The column temperature required for optimal resolution of heteroduplexes was determined empirically for each different amplicon by injection of the control DNA at increasing temperatures until a significant decrease in sample retention time was observed. In addition, the *EXT2* fragment sequences were entered into the WAVEMaker™ program (Transgenomic) which derives the optimal column temperature(s) and gradient for each fragment.

Sequencing analysis

Fragments that showed a variant F-SSCP or DHPLC pattern were further analysed by sequencing. Fragments of interest were PCR amplified from genomic DNA with specific primers (Table 1) and then purified. They were directly sequenced in both directions by use of the BigDye Terminator Cycle

Sequencing Ready Reaction kit (PE-Applied Biosystems) and were run on the ABI 377 DNA Sequencer (PE-Applied Biosystems). Where available, amplicons from one affected and one unaffected member of the family were also sequenced, to check that the mutation was found only in the disease phenotype.

Results

Genomic DNA from 34 unrelated patients with HME was analysed for mutations in the *EXT1* and *EXT2* genes. DNA fragments covering every translated exon with flanking intronic splice site sequence were amplified and screened blindly with both DHPLC and F-SSCP techniques. All variants detected by either method were sequenced; and all were found to have sequence changes.

Forty-two different amplicon variants, listed in Table 2, were detected by blind screening of the *EXT1* and *EXT2* genes. These arise from 37 different sequence changes, due to overlapping amplicon design for *EXT1* exon 1 and *EXT2* exon 2. After the initial screen and sequence analysis of variants, electrophoretic data for the four variants detected

Table 2a Sequence changes in the *EXT1* gene detected by F-SSCP and/or DHPLC

Sequence change	*Fragment no.*	*Distance from nearest end (bp)*	*Detected in blind screen*		*Reason for missing*
			DHPLC	*F-SSCP*	
37delG	1.1a	70	yes	yes	
86–88delG	1.1a	19–21	yes	yes	
86–88 delG	1.1b	54–56	yes	yes	
238delA	1.1b	30	yes	yes	
238delA	1.2	49	yes	yes	
242–247delC	1.1b	21–26	yes	**NO**	mutation not detectable
242–247delC	1.2	53–58	yes	yes	
420ins4	1.2	47	yes	yes	
420ins4	1.3	32	yes	yes	
460–461delTT	1.3	72–73	yes	yes	
T482G	1.3	94	yes	**NO**	human error
713delC	1.4	111	yes	yes	
962 +2 T>C	1.5	39	yes	yes	
C1018T	2	100	yes	yes	
G1019T	2	99	yes	yes	
G1019A	2	99	yes	yes	
C1065T	3	44	yes	yes	
G1066A	3	45	yes	yes	
A1213T	4	110	yes	yes	
1284 +64 G>A	4	26	yes	**NO**	mutation not obvious
1285 –2 A>C	5	70	**NO**	yes	mutation not obvious
1384delC	5	76	yes	yes	
1418 –2 A>G	6	42	yes	yes	
C1431T	6	57	yes	yes	
1463–1468delC	6	89–94	**NO**	yes	human error
1536 +1 G>T	6	69	yes	yes	
1633 –2delA	8	82	yes	yes	
G1761A	9	78	**NO**	yes	human error
A1807T	9	124	yes	yes	
1904–1906delC	10	61–63	**NO**	yes	mutation not obvious

Table 2b Sequence changes in the *EXT2* gene detected by F-SSCP and/or DHPLC

Sequence change	*Fragment no.*	*Distance from nearest end (bp)*	*Detected in blind screen*		*Reason for missing*
			DHPLC	*F-SSCP*	
C28A	2.1	111	yes	yes	
G151T	2.2	104	yes	yes	
450del4	2.3	22	yes	yes	
450del4	2.4	101	yes	yes	
G519C	2.4	59	yes	yes	
A520C	2.4	58	yes	yes	
536 +1 G>A	2.4	41	yes	yes	
C544T	3	67	yes	yes	
G679A	4	111	yes	yes	
1080 –18 T>A	7	29	yes	yes	
1173 +1 G>A	7	60	yes	yes	
1174 –18 G>T	8	24	yes	**NO**	mutation not detectable

by DHPLC but not by F-SSCP technology were re-examined. One of the sequence variants, T482G (Figure 1), did actually show a clear electrophoretic mobility shift relative to control DNA. This shift had initially been disregarded, as it had not been anticipated that this class of peak shift could be caused by a genuine heterozygous sequence alteration. This change was in fact detected when analysed using the Genotyper program (PE-Applied Biosystems). Of the amplicons containing the remaining three variants, one was seen to show a change in electrophoretic mobility that was too subtle to be reliably detected by eye, whilst the other two showed no detectable electrophoretic change.

This procedure was repeated for the four sequence changes detected using F-SSCP but not by the DHPLC technique. Two of these were found to have been missed solely due to human error: in the absence of the WAVEMaker™ temperature prediction facility when analysing *EXT1* fragments, the incorrect temperature for resolution had been chosen. The other two variants were deemed to have peak changes too subtle to be reliably detected using the current scoring method.

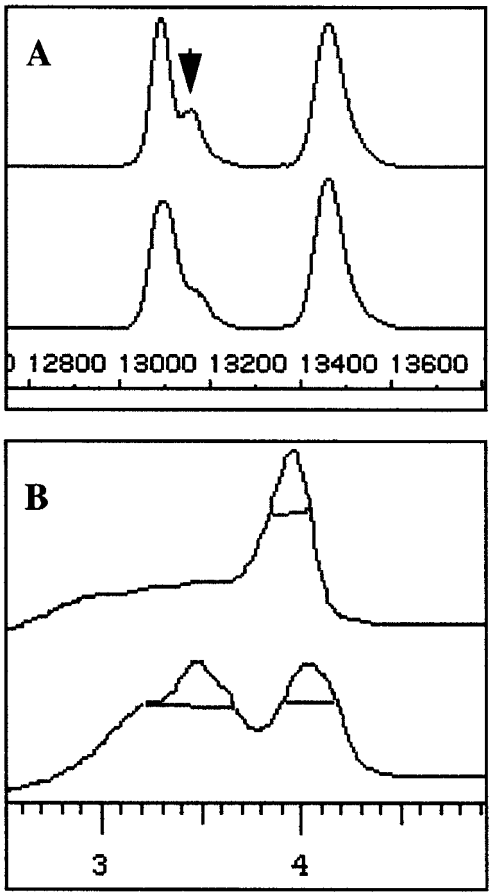

Figure 1 ***A*** Comparison of F-SSCP electropherograms for wild-type sequence (top) and the T482G mutation (bottom) in EXT1 exon 1 fragment 3, aligned and analysed using the Genescan Fragment Analysis program. The x axis represents time, the y axis is a measure of fluorescence. Notice that the minor peak on the top trace (arrowed) is lost on the bottom electropherogram, and that the leftmost peak is reduced in height relative to the right-hand peak. ***B*** Comparison of the DHPLC elution peaks for the same fragments, analysed using the D/7000 HSM program on the WAVE™ DNA Fragment Analysis System.

The detection efficiency rate, assuming that all amplicon variants had been found between the two techniques and discounting human error, was calculated to be 39/42 = 93% for F-SSCP and 40/42 = 95% for DHPLC.

Careful examination of the F-SSCP electropherogram data clearly indicates that the genotype of a fragment can be determined for a known polymorphism, as shown in Figure 2. Furthermore, when fragments containing a common polymorphism were analysed, additional mutations were clearly distinguishable. In the case of DHPLC, we were unable to differentiate between the two homozygous states for a polymorphism; and heterozygotes for new mutations could not be reliably distinguished from heterozygotes at the polymorphic site.

The 37 different sequence alterations found in *EXT1* and *EXT2* were subdivided as follows: three were found in intronic sequence, distant from the splice site; five were silent mutations; and two were conservative mutations. The remaining 27 changes, 21 in *EXT1* and six in *EXT2*, were presumed to be the disease mutation in the respective proband, either because the same mutation had been previously reported to be associated with the HME phenotype, or because the mutation was both deduced to result in an aberrant protein and was not found in an unaffected family member. Of these 27 mutations, 16 in *EXT1* and three in *EXT2* had not been previously reported: these are detailed in Table 3. Six of these were splice-site mutations, eight were insertions or deletions leading to a shift in the reading frame which introduced a premature stop codon, and five were nonsense mutations resulting in the synthesis of a truncated product. No previously unreported missense mutations were found.

Discussion

HME mutations

From 34 probands screened, we found 27 different mutations likely to cause HME in 29 families. Nineteen of these disease mutations have not previously been reported, indicating a strong allelic heterogeneity with no one mutation causing the majority of HME cases in the population. 23/27 (85%) of the mutations detected in this study were loss-of-function mutations, further supporting the theory that *EXT1* and *EXT2* function as tumour suppressor genes. All missense mutations detected in this study had been previously reported,[21,24,25] implying that the two amino acid residues involved, R340 in *EXT1* and D227 in *EXT2*, are essential for maintaining correct protein function or stability.

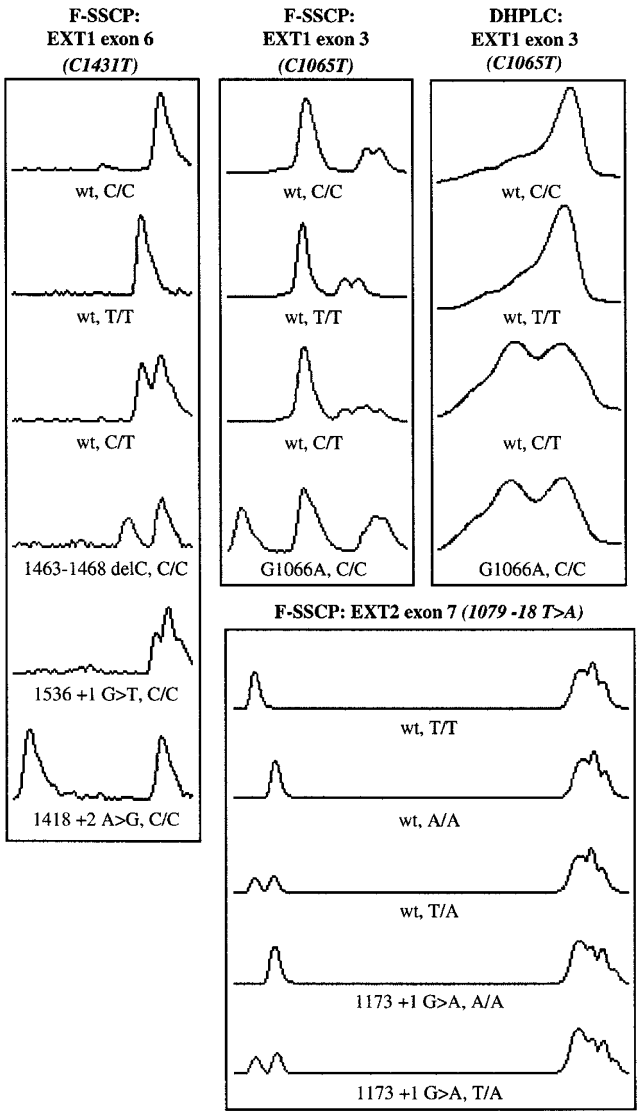

Figure 2 Detection of mutations against a polymorphic background. F-SSCP electropherograms were aligned using the Genescan Fragment Analysis program; DHPLC elution peaks were analysed with the D/7000 HSM program. The detection technique and polymorphism are denoted above the relevant exon group. Each electropherogram is labelled with the disease mutation it represents, followed by the genotype of the polymorphism present. Those electropherograms of fragments with no disease mutation are labelled 'wt'.

Of the 29 families in which mutations were detected, 22 (76%) had a mutation in the *EXT1* gene and seven (24%) in *EXT2*. This contrasts with reported proportional *EXT1*/*EXT2* frequencies of 58%/42% and 50%/50% in similar studies conducted by Philippe *et al*[21] and Wuyts *et al*[22] respectively, although this is probably an effect of the relatively small number of families screened in all three studies. In five families in this study (15%), no mutations in either *EXT1* or *EXT2* were detected. This could be due to several factors: the limitations of the mutation screening techniques used; the fact that 5' and 3' UTRs and promoter regions of the two genes were not analysed; or the existence of other HME-causing genes. The two studies by Philippe *et al* and Wuyts *et al* reported respectively that 29% and 23% of families with the HME phenotype had no detectable *EXT1* or *EXT2* mutations.[21,22] The discrepancy between these figures and ours could be ascribed to the decreased sensitivities of conformation-sensitive gel electrophoresis and conventional SSCP – the respective mutation detection techniques employed by those studies – when compared with our combined F-SSCP and DHPLC strategy.

Comparison of DHPLC and F-SSCP

The mutation detection rates for DHPLC and F-SSCP, as determined in this study, are very similar: 95% and 93% respectively. These figures are not absolute, as it is not possible to rule out the occurrence of sequence variants that have passed undetected by either technique without sequencing all 1088 fragments in both directions. It is reasonable, however, to conclude that both methods are capable of high sensitivities. Looking in detail at the base changes undetected by F-SSCP, it can be seen that they are all positioned <30 bp from the amplicon ends. This is in contrast to results from work with conventional SSCP, where position of base change has been reported to play little role in sensitivity.[26–28] It follows that judicious design of primers sufficiently far from the region of interest will further increase mutation detection using this technique. Looking at the changes deemed undetectable by DHPLC reveals no such obvious trend: a larger study would be necessary for drawing any conclusions about the nature of 'missed' variants. This study has only assayed the detection efficiency for fragments within the optimal size range recommended for SSCP, ie 150–300 bp.[29] It is known that conventional SSCP sensitivity drops sharply for fragments larger than 300 bp.[29,30] This is in contrast to DHPLC, where single base mutations have been detected in fragments as large as 1.5 kb,[31] although manufacturers of the integrated system recommend that for optimal sensitivity, amplicons should be kept to a maximum of 450 bp.[4]

Also of interest when considering screening strategies is the ability of a particular technique to determine the genotype of a single nucleotide polymorphism (SNP). As shown in Figure 2, it is possible to distinguish between all three genotypes for the common SNPs encountered in this study when using F-SSCP. In contrast, the two homozygous states for the SNPs cannot be differentiated from each other using the DHPLC protocol detailed here. This could be simply achieved by comparison of the data with that gleaned from prior mixing of the fragments with a homozygous reference sample, although this would increase running and analysis time. A related concern is whether a technique is capable of distinguishing between different mutations in the same

fragment. For example, *EXT1* exon 3 contains a C/T polymorphism at position 1065 (cDNA numbering). One individual in our study was homozygous for this polymorphism, but was heterozygous for the G1066A mutation immediately adjacent. As shown in Figure 2, these two sequence variants can be clearly distinguished by F-SSCP: this is not the case with DHPLC. This may have implications for the utility of the latter technique in mutation screening if there is a relatively common polymorphism in the region of interest.

Sensitivity of detection is not the only factor that must be taken into account when evaluating techniques that identify sequence variants. Throughput, ease of application and cost are all elements that must be considered in the choice of a screening technique.

Throughput In this study, 185–222 amplicons were routinely screened in one 8 h F-SSCP electrophoresis. It is envisaged that multiplexing 9 fragments in one lane will be practically viable, enabling the running of up to 576 fragments in a single electrophoresis when using a 64-well gel. With present technology, it was possible to screen only 49 amplicons in 8 h using DHPLC. However, equipment is available that allows faster column cleaning and equilibration between injections, which could substantially increase throughput.

Ease of application Both F-SSCP and DHPLC are relatively simple techniques, involving standard processes. PCR amplification is perhaps easier with F-SSCP because a standard *Taq* polymerase is sufficient for the reaction. This is not the case with DHPLC: this technique is susceptible to errors introduced in PCR and therefore requires the presence of a proofreading polymerase. Unfortunately, these polymerases are more sensitive to DNA quality, which resulted in repeat amplifications being required for some samples in this study. However, once DNA fragments have been amplified, DHPLC is far quicker to set up, as it includes no time-consuming purification or gel-pouring steps.

Cost The protocol for DHPLC employed in this study is calculated to be £0.79 per amplicon, assuming the process is run at only one temperature. (An additional temperature is often required, depending on the melting profile of the DNA fragment: this costs an extra £0.33 per amplicon). The F-SSCP protocol used here is calculated to cost £0.61 for one fragment when run under the two electrophoresis conditions. However, this calculation assumes full consumption of the fluorescent primers, which is unlikely to be the case when screening only a few individuals. This initial cost, which can rise greatly if screening many different exons, can be circumvented by the use of an alternative protocol, post-label F-SSCP.[32] This technique involves amplification of the DNA region of interest using unlabelled primers with 5' ATT extensions, then performing a Klenow fragment nucleotide exchange reaction with fluorescently-labelled dUTPs. This has proved successful in our hands and should theoretically result in no loss of sensitivity, although this has not been thoroughly tested.

In summary, both DHPLC and F-SSCP display very high mutation detection rates in this study. The main strengths of DHPLC are its extreme ease of application and analysis and its relatively low cost. This makes this technology ideal for screening large regions of the genome in relatively few individuals for single nucleotide polymorphisms, or for screening potential candidate genes for mutations associated with the disease in question. The main advantages associated with F-SSCP are its capacity for extremely high throughput and its ability to distinguish between different mutations in the same fragment. It is therefore ideally suited for screening a large number of individuals for mutations in a known disease gene that has relatively few exons. With this in mind,

Table 3 Previously unreported mutations identified in the *EXT1* and *EXT2* genes

cDNA change	*Gene*	*Exon or intron*	*Protein change*	*Type of mutation*
37delG	EXT1	Exon 1	FS	Frameshift
86–88delG	EXT1	Exon 1	FS	Frameshift
238delA	EXT1	Exon 1	FS	Frameshift
242–247delC	EXT1	Exon 1	FS	Frameshift
460–461delTT	EXT1	Exon 1	FS	Frameshift
T482G	EXT1	Exon 1	L161X	Nonsense
962 +2 T>C	EXT1	Intron 1	?	Splice site
A1213T	EXT1	Exon 4	R405X	Nonsense
1285 –2 A>C	EXT1	Intron 4	?	Splice site
1384delC	EXT1	Exon 5	FS	Frameshift
1418 –2 A>G	EXT1	Intron 5	?	Splice site
1463–1468delC	EXT1	Exon 6	FS	Frameshift
1536 +1 G>T	EXT1	Intron 6	?	Splice site
1633 –2delA	EXT1	Intron 7	?	Splice site
A1807T	EXT1	Exon 9	K603X	Nonsense
1904–1906delC	EXT1	Exon 10	FS	Frameshift
G151T	EXT2	Exon 2	E51X	Nonsense
536 +1 G>A	EXT2	Intron 2	?	Splice site
C544T	EXT2	Exon 3	R181X	Nonsense

we have decided to continue with F-SSCP in our screening strategy for mutations in *EXT1* and *EXT2*.

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