

An Alu-mediated partial *SDHC* deletion causes familial and sporadic paraganglioma

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Hereditary paraganglioma (PGL) is characterised by slow growing, vascular tumours that can develop in any component of the paraganglia, a neuro-ectodermal system that is distributed from the skull base to the pelvic floor.¹ Common tumour sites include the carotid body in the head and neck and adrenal and extra-adrenal paraganglia in the abdomen. Heterozygous germline inactivating mutations in *SDHD*, *SDHC*, and *SDHB*, which encode three of the four subunits of mitochondrial complex II (succinate dehydrogenase), cause hereditary paraganglioma types 1, 3, and 4 (PGL1, PGL3, and PGL4), respectively. Mutations in the fourth subunit of mitochondrial complex II, *SDHA*, have yet to be demonstrated in hereditary paraganglioma. Germline loss of function mutations followed by somatic loss of non-mutant alleles in the tumours^{2–4} suggests a tumour suppressor role for mitochondrial complex II in the paraganglia.

Over 25 mutations in *SDHD* and 25 mutations in *SDHB* have been detected in hereditary paraganglioma, including those reviewed by Baysal¹ and the more recent additions of multiple mutations in *SDHB*^{4–6} and *SDHD*.^{7–9} All reported mutations are single nucleotide alterations leading to splice site, missense, nonsense, or frameshift mutations, or intra-exonic deletions and insertions of up to four nucleotides, which have been detected through exonic PCR amplifications and sequencing. In contrast to the abundance mutations in *SDHB* and *SDHD*, only a single multiply affected family and an isolated case, containing a single nucleotide initiation codon and a splice site mutations in *SDHC*, respectively, have been described by Niemann *et al.*^{3,10} However, analyses of *SDHC* in four series of patients with paraganglioma or pheochromocytoma^{6,8,11,12} yielded no definitive *SDHC* mutations. These findings indicate that the relative contribution of complex II subunit mutations to hereditary paraganglioma is not similar and may reflect currently unrecognised aspects of complex II biology. Hence, it is of utmost importance that role of *SDHC* in familial and sporadic paragangliomas be confirmed independently.

Penetrance of complex II mutations shows peculiar characteristics. Mutations in *SDHD* cause PGL1 only if the transmission occurs paternally, whereas maternal transmission does not cause disease,¹³ suggesting operation of genomic imprinting on *SDHD*. In contrast, *SDHB* mutations are transmitted both paternally¹¹ and maternally.¹⁴ Thus far, transmissions of *SDHC* mutations causing disease occurred through mothers in the one multiplex family¹⁴ and in one isolated case¹⁰. Because the molecular basis of the parent of origin effects in PGL1 is unknown, it is unclear whether transmissions of mutations in *SDHC*, the protein product of which couples with that of *SDHD* and forms the membrane spanning domain of mitochondrial complex II, also shows any parent of origin effects.

METHODS

The family and the isolated cases were ascertained from two US otolaryngology clinics (University of Pittsburgh School of

Key points

- Hereditary paraganglioma (PGL) is characterised by the development of vascularised tumours in the head, neck, and abdomen and is caused by germline heterozygous inactivating mutations in mitochondrial complex II succinate dehydrogenase (SDH) genes *SDHB* (hereditary paraganglioma type 4 (PGL4)), *SDHC* (PGL3), and *SDHD* (PGL1). *SDHD* mutations cause PGL1 after paternal, but not maternal, transmissions, which suggests genomic imprinting. Mutation analyses in several familial paraganglioma series uncovered many conventional mutations in *SDHB* and *SDHD* but failed to detect *SDHC* mutations. So far, only a single multiplex PGL3 family with a missense initiation codon mutation, that is transmitted maternally, has been described.
- We analysed a family with head and neck paragangliomas and discovered an 8.37 kb *SDHC* deletion, which spans two AluY elements and removes exon 6. The deletion caused PGL3 following both maternal and paternal transmissions in the pedigree and was also detected in an unrelated sporadic case who showed allele sharing with the familial cases at seven polymorphic markers near *SDHC*, suggesting a common ancestral origin.
- These findings, for the first time, to our knowledge, describe a large deletion in a complex II gene and confirm the role of *SDHC* in familial and sporadic paragangliomas. The observation of both paternal and maternal disease transmissions in PGL3, together with earlier findings, suggests that imprinted transmission in hereditary paraganglioma is restricted to *SDHD* among complex II genes.

Medicine, Pittsburgh, PA and House Ear Institute, Los Angeles, CA) under research protocols approved by the University Institutional Review Board committee. DNA isolation, genotyping of simple tandem repeat polymorphisms, PCR amplification and sequencing were performed using standard techniques² and all simple tandem repeat polymorphisms were amplified in the presence of 10% glycerol and 5% DMSO after labelling one oligonucleotide primer with ³²P and analysed on a 6% polyacrylamide gel. DNA from the multiplex family was isolated either directly from white blood cells or from transformed lymphoblastoid

Abbreviations: PGL1, PGL3, and PGL4, hereditary paraganglioma types 1, 3, and 4; PLNA, percentage loss of normal alleles; RA, ratio of alleles; SDH, succinate dehydrogenase

cell lines. DNA of the sporadic cases was isolated from cheek swabs as described earlier.¹¹

The separation of chromosome 1 for *SDHC* mutation analysis in somatic cell hybrids was performed commercially (GMP Genetics, Waltham, MA), using a conversion approach that employs fusion between human and rodent cells to create stable hybrids that contain only a subset of the human chromosomes. This approach significantly increases the sensitivity to detect unconventional mutations that could be missed by techniques based on PCR because the parental copies of a given chromosomal pair can be separated and tested individually.¹⁵ An EBV transformed lymphoblastoid cell line derived from an affected individual (4-2, fig 1) was used for chromosomal separation by the conversion approach. This subject was chosen for chromosome separation because he is affected and an obligate carrier of the familial mutation (fig 1).

Three independent somatic cell hybrids for the disease chromosome 1 and three independent somatic cell hybrids for the normal chromosome 1 were obtained from subject 4-2 and tested by sequence tagged site analysis. In addition to the *SDHC* exon 6,¹¹ the following PCR primer pairs located near *SDHC* exon 6 amplified a product at the expected size (given in parenthesis) from the hybrids containing the normal chromosome 1 but did not amplify from the hybrids

containing the disease chromosome 1: (1) 120F: 5'-TTGGATCGCCCCTGGGCT-3' and 120R: 5'-ACAAGCATAGCTCTCAGGGT-3' (512 bp); the amplicon is located ≈5 kb downstream of exon 6; (2) 123F: 5'-TTGTCTTCAGTTGGTATGCCT-3' and 123R: 5'-GAAGATTCTGGAAGGAGACAC-3' (243 bp); the amplicon is located ≈7 kb downstream of exon 6. In addition to the *SDHC* exon 5,¹¹ the following PCR primer pairs located near *SDHC* exon 6 amplified a product of the expected size (in parenthesis) from the hybrids containing both the disease and the normal chromosomes 1: (1) 124F: 5'-GAACTAATTGATTGAACTAGTAG-3' and 124R: 5'-CCATGTTTAACCTACAGCTTAAC-3' (236 bp); the amplicon is located ≈8.5 kb downstream of exon 6; (2) 110FB: 5'-GGAGAAAATATATGTTTTTAAATGAAG-3' and 110R: 5'-CAGTCAATCTCAGAATCTTT-3' (283 bp); the amplicon is located ≈1.3 kb upstream of exon 6. On the basis of these findings, we attempted to PCR amplify the deleted allele using oligonucleotide primer pairs that are too far apart to amplify the normal genomic DNA by standard PCR.

The PCR primers, 112F: 5'-CCTTAGAATACTAGTCC TCTGA-3' and 124R: 5'-CCATGTTTAACCTACAGCTTAAC-3', which are located 9405 bp apart (including primer binding sequences) in the normal genomic sequence (GenBank accession number AL592295) and span *SDHC* exon 6, captured the deletion junction in a 1037 bp fragment

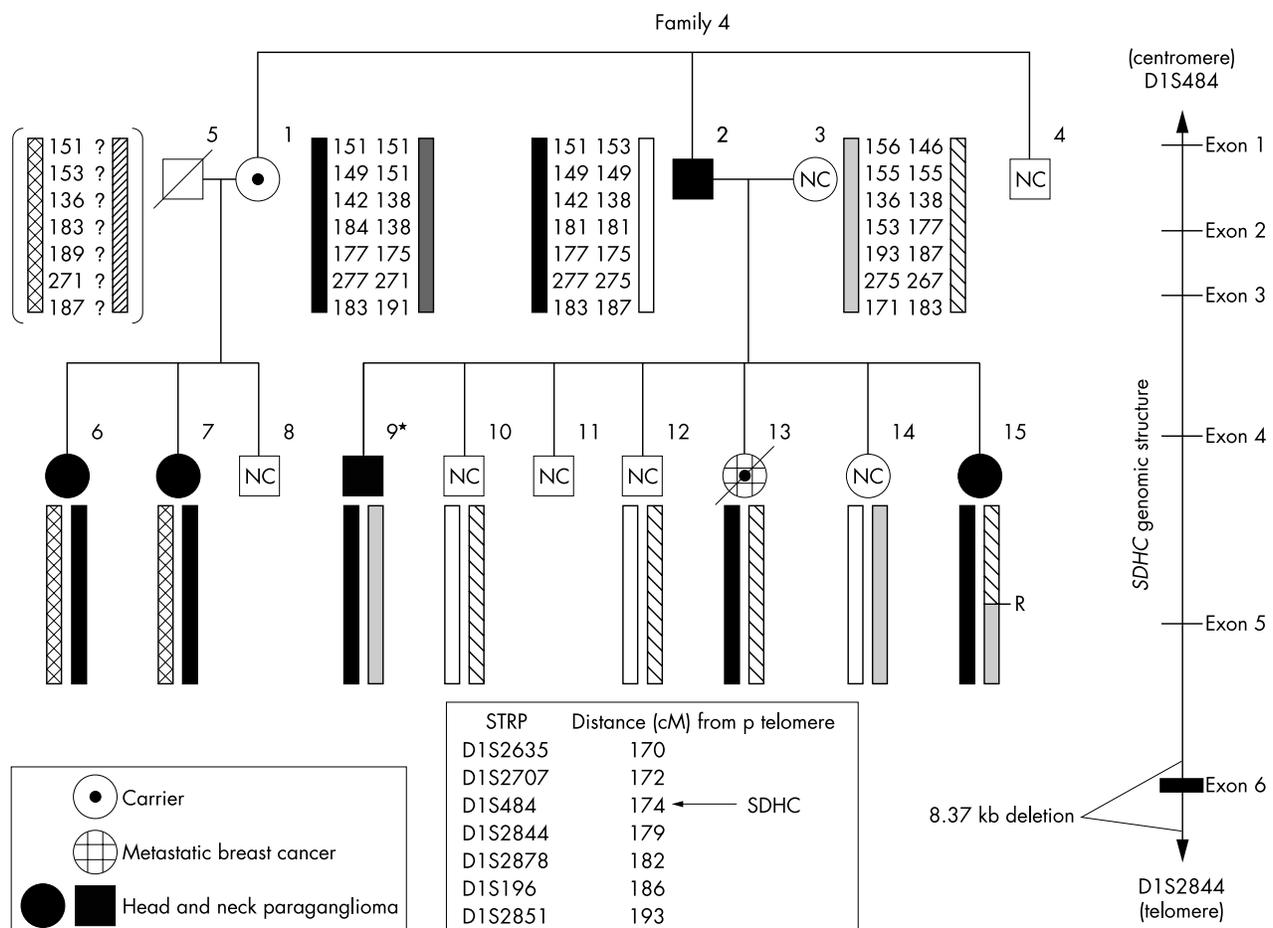


Figure 1 A simplified pedigree of the multiplex family with *SDHC* deletion. Differently patterned long vertical bars symbolise individual haplotypes spanning the *SDHC* gene. Tested simple tandem repeat polymorphisms around the *SDHC* gene are shown in a box and the alleles (in bp) segregating in the family are depicted for subjects 1, 2, and 3. A haplotype of a married and deceased subject (5) was inferred and is shown in parenthesis. A diagram depicting the *SDHC* genomic structure, its relative position to two flanking simple tandem repeat polymorphisms (STRP) (D1S484 and D1S2844) and the location of the deletion identified in this study is shown on the right. D1S484 is located ≈500 kb centromeric to *SDHC*. Pathology records for subject 9 (shown by an asterisk) and for an unrelated sporadic subject with an identical *SDHC* deletion were available. R, a recombination event in subject 15 between D1S2844 and D1S196. NC, non-carrier of the deletion spanning *SDHC* exon 6.

Table 1 Phenotypic characteristics of affected and carrier subjects

Subject	Age at symptom onset	Summary of head and neck paraganglioma findings	Associated conditions
1, carrier*	No symptom at age 72	No tumour detected	Stroke, carotid artery surgery
2, affected*	20	Large unilateral carotid body tumour (not resected), visible, palpable lump in the neck	-
6, affected*	42	Unilateral carotid body tumour (not resected), visible, palpable lump in the neck, difficulty swallowing	"Thyroid tumour" resected at age 24, lump in breast at age 48
7, affected*	40	Unilateral carotid body tumour (resected),	-
9, affected†	20	Unilateral carotid body tumour (resected and confirmed by histology)	-
13, carrier	Not applicable	Not applicable	Died of metastatic breast cancer at age 44
15, affected*	13	Carotid body tumour (resected), lost voice in teenage years	-
Sporadic case, affected†	45	Vagal and hypopharyngeal paraganglioma (resected and confirmed by histology)	-

*Information obtained through medical history and interview.

†Information obtained through medical history and interview is also confirmed by pathology and surgery reports.

(including a four base pair non-templated junctional insertion). The PCR amplification was performed for 38 cycles after an initial 10 min denaturation at 94°C. Each cycle was composed of 45 s incubation at 94°C, 45 s incubation at an annealing temperature of 54°C and 2.5 min incubation at 72°C for extension. The reaction was terminated with a final 7 min of extension period. AmpliTaq Gold Taq polymerase enzyme was used in all PCR amplifications.

The degree of allelic loss was assessed by comparison of the intensities of parental alleles between the tumour sample and peripheral blood as described.¹⁶ Allele intensities were quantified after densitometric scanning of the x ray autoradiograms using VIDEK Harmony Bioscan Software (v 4.03, Aldus). We first calculated the ratio of alleles, $RA = (\text{normal allele/disease allele})$ in blood (RA_b) and in tumour (RA_t). We then calculated the percentage loss of normal alleles (PLNA) in the tumour by $PLNA = 100 \times [1 - (RA_t/RA_b)]$. For example, if there is no relative loss of normal allele in the tumour (that is, $RA_t = RA_b$), then the percentage loss of normal alleles equals

0%. If there is a complete loss of normal allele in the tumour, then the percentage loss of normal alleles equals 100%.

The following web based resources were used in this study:

- RepeatMasker program at <http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html>;
- Blast 2 program at www.ncbi.nlm.nih.gov/BLAST/;
- UCSC genome database at www.genome.ucsc.edu/, which contains the full genomic sequence of the *SDHC* gene.

RESULTS AND DISCUSSION

Previously, we reported the presence of germline mutations in *SDHB* and *SDHD* in 70% (7/10) of familial and $\approx 8\%$ (3/37) of non-familial clinic patients with head and neck paragangliomas but no mutations could be identified in the *SDHC* gene.¹¹ Here, we report discovery of a *SDHC* mutation in one of the remaining three families, family 4.^{11,16} We have evaluated family 4 by recruiting additional members (fig 1). The detailed phenotype of the affected subjects from this

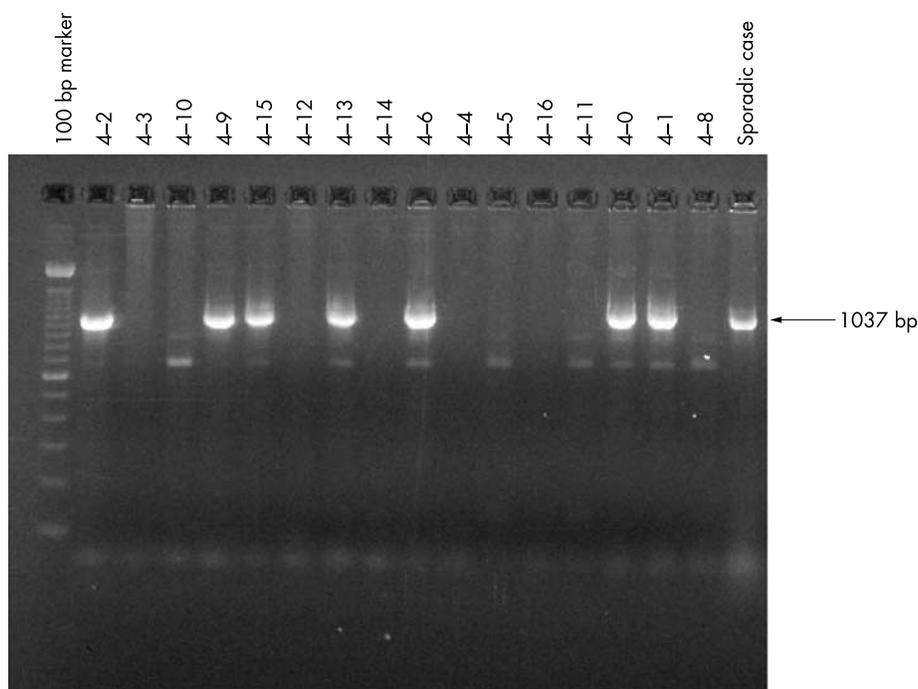


Figure 2 Genomic amplification of the deletion junction in the members of the multiplex family and in the isolated case as a 1037 bp fragment. The numbers above the lanes correspond to the subjects in the pedigree (fig 1).

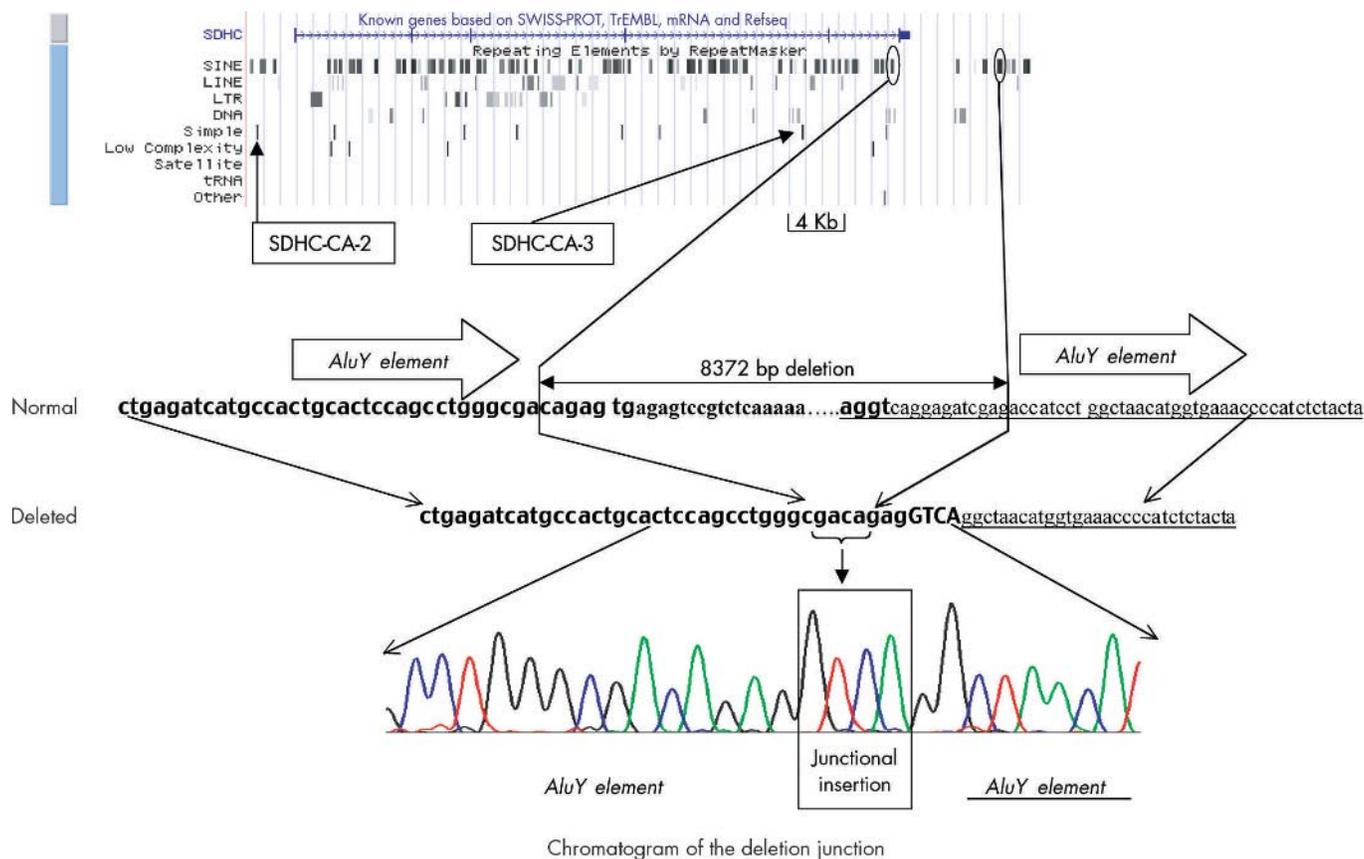


Figure 3 The upper panel shows genomic structure and high repeat element content of the *SDHC* gene (data from UCSC database). Vertical bars in the *SDHC* genomic sequence indicate six exons of the gene. The deletion occurred between two *AluY* elements (circled) that span exon 6. The two *AluY* elements have the same genomic orientation (arrows). Locations of two (CA/GT)_n repeat elements, *SDHC-CA-2* and *SDHC-CA-3*, used in haplotype and allelic imbalance analyses are indicated by arrows (table 2). The lower panel shows nucleotide composition around the deletion breakpoints and the sequence chromatogram of the deletion junction. The sequence of the telomeric *AluY* element is underlined. Four nucleotides shown in capitals at the deletion junction were non-templated insertions (filler DNA). SINE, short interspersed repeat elements, including *Alus*; LINE, long interspersed repeat elements; LTR, long terminal repeats.

family is listed in table 1. The information on phenotype of most affected subjects was obtained through their medical histories, although pathology reports, confirming diagnoses of paragangliomas, were also available for two subjects. Both paternal and maternal disease transmissions were observed in the pedigree suggesting that *SDHD* was not the underlying locus. No subject reported having been diagnosed with abdominal or metastatic paragangliomas, which may be associated with *SDHB* mutations.^{4 14 17} We reasoned that either a mutation in a new hereditary paraganglioma gene or an unconventional mutation in the known hereditary paraganglioma genes was responsible for the disease.

To test for cosegregation of alleles among the affected subjects we genotyped simple tandem repeat polymorphisms near *SDHB*, *SDHC*, and *SDHD* genes. We found that haplotypes defined by simple tandem repeat polymorphisms near *SDHB* and *SDHD* genes did not cosegregate among the affected subjects, further excluding the role of these genes in this family (data not shown). However, multimarker haplotypes spanning *SDHC* were consistently shared among the five affected subjects (fig 1). Haplotype analysis further suggested the presence of two more mutation carriers, including an obligate carrier mother and a subject who died of metastatic breast cancer (fig 1). Neither carrier was clinically diagnosed with hereditary paraganglioma (table 1). The obligate carrier mother reported undergoing carotid artery surgery following an episode of stroke but denied ever

been diagnosed with a head and neck tumour. The phenotypic information for both carriers was obtained through interviews; their medical records were not available for detailed investigation.

Because simple tandem repeat polymorphisms flanking *SDHC* were consistent with linkage, we hypothesised that a genomic rearrangement that escapes detection by PCR amplification of the exons might be responsible for PGL3 in this family. An amplification of *SDHC* cDNA prepared from lymphoblastoid total RNA from an affected subject by RT-PCR, using expressed oligonucleotide primers from exon 1 and exon 6,¹¹ revealed only the expected normal transcript size without any evidence of an aberrant band. To obtain direct evidence for gross gene alterations, we pursued separation of the disease and non-disease chromosomes to perform sequence tagged site content analysis. The *SDHC* gene is localised at the long arm of chromosome 1 at band q23.3 at the UCSC genome database, which is far more distal than was reported earlier.¹⁸ Following the separation of the two parental chromosomes 1 in somatic cell hybrids, we conducted sequence tagged site content analysis using three stable hybrids for each parental chromosomes 1 derived from subject 4-2 (fig 1). We found that oligonucleotide PCR primer pairs that span *SDHC* exon 6 did not amplify from any of the three hybrids containing the disease chromosome, suggesting a deletion spanning the 3'-end of the gene. Further sequence tagged site content mapping around exon 6 confirmed the deletion and enabled us to capture a junctional PCR fragment

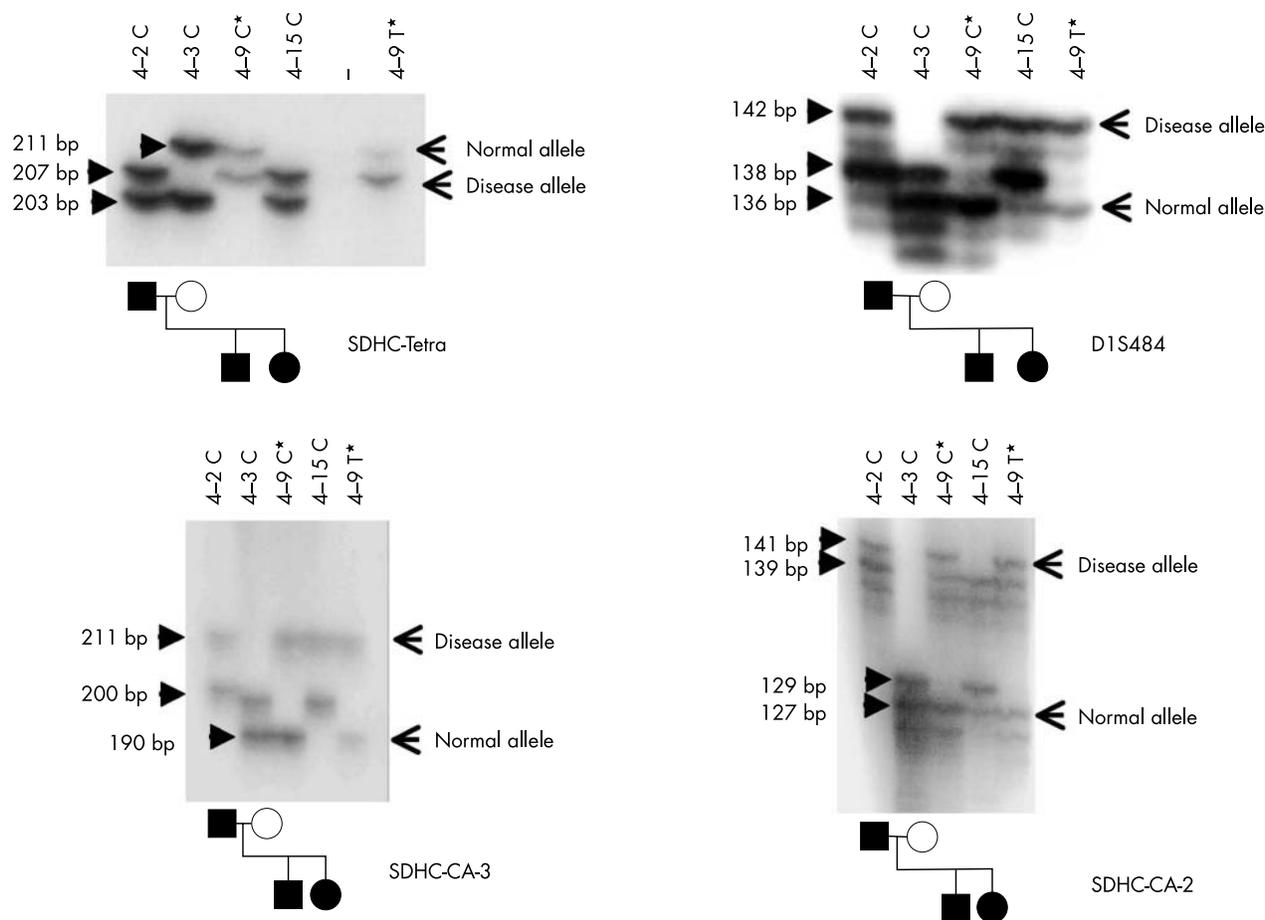


Figure 4 Partial somatic losses of normal alleles in hereditary paraganglioma tumour from subject 4-9 for four simple tandem repeat polymorphisms near the *SDHC* gene. Comparison of relative intensities of the normal alleles between the constitutional (C) and tumour (T) DNAs from subject 4-9 (in lanes denoted by an asterisk) demonstrates partial losses of the normal alleles in the tumour for all markers (denoted by arrowheads). Genotypes of certain pedigree members (fig 1) are included to demonstrate the normal and disease alleles segregating in the pedigree. Note that affected subject 4-15 did not share *SDHC*-CA-2 disease allele (141 bp) with the other affected individuals in the pedigree. This was most probably caused by a spontaneous mutation of the STRP allele because subject 4-15 shared the disease alleles of other simple tandem repeat polymorphisms (fig 1) and was a carrier of the *SDHC* deletion (fig 2). Sizes (in base pairs) of the segregating alleles (denoted by filled arrowheads) are indicated for each marker.

which amplified from the five affected and the two carrier subjects (fig 2), as predicted by haplotype analysis, but not from the other unaffected family members and 103 additional control subjects. The deletion was heterozygous in all six carriers since the normal copy of *SDHC* exon 6 could be amplified from constitutional genomic DNA.

Sequence analysis of the junctional fragment indicated that an 8372 bp genomic fragment spanning exon 6 was deleted (fig 3). Both breakpoints mapped within AluY elements, which are normally located 8.25 kb apart on the

genomic sequence in identical orientations. An alignment by the “Blast 2” program indicated an 84% sequence identity between the two AluY elements. We also detected a non-templated insertion of four nucleotides at the deletion junction (that is, filler DNA). Filler DNAs are random insertions at the breakpoint junctions of constitutional and somatic chromosomal rearrangements and are incorporated through a variety of mechanisms including non-homologous end repair.¹⁹ The adjoining of two similar AluY elements by the deletion and the identification of a 4 bp insertion at the

Table 2 New STRPs near *SDHC* gene

Locus/repeat motif	Location	Primer sequences (5' to 3')	Allele no./range (bp) Expected heterozygosity
<i>SDHC</i> -CA-2/(Ca)n	3 kb upstream of exon 1	GCAAAAAGCAAGGACAAAGAGGT TATATCCAACACTTCAGAAACCT	10/127–147 0.85
<i>SDHC</i> -CA-3/(Ca)n	Intron 4	TTATGGGAAACAACCTGGCCTCT GCAGGAAGCTGAGATCGCA	4/190–211 0.66
<i>SDHC</i> -3'-STRP/complex (TA)n(GA)n	33.5 kb downstream of exon 6	CAGTGAGCCGAGATCAATGG TGACCAACAGGGCCTGCT	6/150–160 0.72
<i>SDHC</i> -Tetra/(GAAA)n	68 kb downstream of exon 6	ACTCCAGTCTGGGCTGGAGCA TGAGTTTCTGGATTCTACAGA	6/195–219 0.79

junction suggests that a homologous Alu-Alu recombination between two highly similar elements, that occurred intra-chromosomally or interchromosomally, and an ensuing non-homologous end repair was responsible for this genomic rearrangement. The deletion of exon 6 is predicted to remove the third transmembrane spanning domain of the *SDHC* protein product, cybL, as well as the RNA polyadenylation and termination signals.¹⁸

Availability of paraffinized tumours from individual 9 allowed us to test for allelic imbalance near the *SDHC* gene. We tested four simple tandem repeat polymorphisms, *SDHC*-tetra, *SDHC*-CA-2, D1S484, and *SDHC*-CA-3, and found losses of normal alleles in the tumour of 66%, 30%, 38%, and 73%, respectively (fig 4). The lost alleles were located on the non-disease chromosome for each marker, suggesting that the normal copy of *SDHC* is somatically lost during tumour genesis. These results are in accord with those of Niemann and Muller³ and suggest that *SDHC* gene is subject to two hit inactivation consistent with a tumour suppressor role in human paraganglionic tissue.

To test whether this deletion might be present among isolated cases with solitary head and neck paragangliomas who previously failed to reveal any mutations in the *SDHB*, *SDHC*, and *SDHD* genes,¹¹ we conducted PCR analyses in 31 cases. We observed one subject, whose constitutional DNA from cheek swab and blood amplified a fragment at a similar size to that of the familial cases. Direct sequencing of the junctional PCR fragment from the isolated case revealed the same sequence found in the family, including the four base pair filler DNA insertion at the junction. To further assess the possibility of a common origin, we genotyped three previously described and four novel STRP markers that we characterised near the *SDHC* gene (fig 1 and table 2) and found that the sporadic case carried all the alleles located on the disease haplotype in the multiplex family. The tested simple tandem repeat polymorphisms, the shared disease alleles and their frequencies were as follows: D1S2635: 151 bp (0.160), D1S2707: 149 bp (0.130), D1S484: 142 bp (0.107), *SDHC*-CA-2: 141 bp (0.053), *SDHC*-CA-3: 211 bp (0.50), *SDHC*-3'-STRP: 151 bp (0.44), and *SDHC*-tetra: 207 bp (0.143).

The identity of the deletion junctions and the sharing of multiple uncommon alleles of simple tandem repeat polymorphisms distributed over a 4 cM distance strongly suggest that the family and the isolated case inherited the *SDHC* deletion from a common ancestor, who was not apparent by investigation of both extended pedigrees. Geographical proximity of residential locations of the familial and sporadic cases (both from the northeastern United States) further supports this conclusion. Multiple founder mutations have been detected for *SDHD*, but not for *SDHB*.^{11 20 21} Whether the *SDHC* deletion is a founder mutation and might be responsible for additional HNP cases in the United States or elsewhere will be appreciated better by further testing of additional cases. Nevertheless, our findings clearly indicate that *SDHC* mutations contribute to the aetiology of familial and sporadic head and neck paragangliomas. Cosegregation of the *SDHC* deletion with head and neck paragangliomas in five affected subjects in the multiplex family and in one unrelated sporadic case provides the strongest association between a *SDHC* mutation and head and neck paragangliomas so far. For comparison, the initiation codon mutation described in the original PGL3 family was detected in only five subjects with head and neck paragangliomas.³

SUMMARY

Current data further expand the role of complex II mutations in the aetiology of paragangliomas and together with our previous findings¹¹ suggest that *SDHD*, *SDHB*, and *SDHC*

germline mutations contribute to 50%, 20%, and 10% of the familial and $\approx 5\%$, $\approx 3\%$, and $\approx 3\%$ of the sporadic cases with head and neck paragangliomas, respectively. To our knowledge, the *SDHC* deletion described in here is the first large gene deletion identified in a hereditary paraganglioma gene. The discovery of a large deletion that was missed by conventional mutation screening methods may warrant inclusion of screening methods for identifying large deletions. A repeat content analysis of the genomic sequence of *SDHC*, which spans 48 809 bp from the transcription start site to the end of exon 6 in the USCS database, by the RepeatMasker program blocks 42.31% of the sequence as repeat elements and 37% as Alu elements (fig 3). This density of Alu elements is comparable to that of *BRCA1*,²² which has 41.5% Alu content and which undergoes recurrent genomic deletions.²³ It is also well known that other genes with a high Alu repeat content, such as those encoding the LDL receptor and α -globin, are prone to recurrent genomic deletions through Alu-Alu recombination events.²³ Thus, on the basis of its high Alu content, our discovery of an Alu mediated deletion and the failure to find conventional mutations in several paraganglioma series, the *SDHC* gene might be considered further for genomic deletions in familial and sporadic paragangliomas. Because the *SDHC* deletion caused tumours after both paternal and maternal transmissions, it is unlikely that an absolute parent of origin effect operates at the *SDHC* locus. This observation, together with the earlier results on the transmission of *SDHB* mutations, strongly suggests that the parent of origin effect on the *SDHD* gene is not a functional consequence of complex II mutations but a locus specific epigenetic phenomenon operating exclusively on the *SDHD* gene at chromosomal region 11q23.

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SHORT REPORT

A new locus for hereditary haemorrhagic telangiectasia (*HHT3*) maps to chromosome 5

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Patients with hereditary haemorrhagic telangiectasia (HHT, or Osler-Weber-Rendu syndrome) have variable presentation patterns and a high risk of preventable complications. Diagnostic tests for mutations in *endoglin* (HHT type 1) and *ALK-1* (HHT type 2) are available. Some HHT patients are now known to have HHT-juvenile polyposis overlap syndrome due to *Smad4* mutations. Families were ascertained following the presentation of probands for embolization of pulmonary arteriovenous malformations. Genome-wide linkage studies using over 700 polymorphic markers, and sequencing of candidate genes, were performed. In a previously described HHT family unlinked to *endoglin* or *ALK-1*, linkage to *Smad4* was excluded, and no mutations were identified in the *endoglin*, *ALK-1*, or *Smad4* genes. Two point LOD scores and recombination mapping identified a 5.4 cM *HHT3* disease gene interval on chromosome 5 in which a single haplotype was inherited by all affected members of the pedigree. The remainder of the genome was excluded to a 2–5 cM resolution. We are currently studying a further family potentially linked to *HHT3*. We conclude that classical HHT with pulmonary involvement can result from mutations in an unidentified gene on chromosome 5. Identification of *HHT3* should further illuminate HHT pathogenic mechanisms in which aberrant transforming growth factor (TGF)- β signalling is implicated.

Hereditary haemorrhagic telangiectasia (HHT, also known as Osler-Weber-Rendu syndrome) is one of the most common autosomal dominant disorders, affecting between 1 in 5000 and 1 in 8000 people in Europe and Japan.^{1,2} HHT is a genetically heterogeneous group of disorders that lead to common vascular phenotypes. HHT types 1 and 2 have been recognised for more than a decade. HHT1 (OMIM 187300) results from mutations in *endoglin*³ on chromosome 9, whereas the disease gene for HHT2 (OMIM 600376) is *ALK-1* on chromosome 12.⁴ In addition, mutations in *Smad4/MADH4* causing a juvenile polyposis/HHT overlap syndrome (JPHT; OMIM 175050) have been described recently.⁵ Although the existence of a third ‘‘pure’’ HHT locus has been suggested twice,^{6,7} the first family were subsequently demonstrated to have an *ALK-1* mutation,⁸ and further data on the family described by Wallace and Shovlin⁷ have not been presented.

All forms of HHT result in the development of abnormal blood vessels including telangiectasia of the oral mucous membranes, nose, and gastrointestinal tract, and visceral arteriovenous malformations (AVMs). Nosebleeds and chronic gastrointestinal bleeding leading to iron deficiency anaemia and transfusion dependence are the features of HHT most appreciated by clinicians. Visceral AVMs are usually silent, but screening programmes indicate that pulmonary

AVMs occur in 30–50% of HHT patients,^{9,10} cerebral AVMs in 10%,⁹ and hepatic AVMs in 20–30%.^{6,11} Pulmonary AVM-induced embolic strokes and brain abscesses, and cerebral AVM-induced haemorrhagic strokes make HHT a common cause of inherited stroke in young adults,¹² and complications from other visceral involvement, including hepatic failure, also occur. There are subtle differences in the phenotype between HHT1 and HHT2, with HHT2 patients exhibiting fewer pulmonary AVMs and a milder HHT phenotype,¹³ but carrying a higher risk of development of HHT related pulmonary arterial hypertension¹⁴ (table 1).

Importantly, many of the complications of HHT can be prevented or limited by clinical screening programmes. Since HHT is a disease with late onset penetrance ($\approx 90\%$ by 40 years; 97% by 60 years¹⁵), genetic screening programmes have been introduced.¹⁶ Patients without detectable mutations in *endoglin* or *ALK-1* are recognised by the HHT genetic centres. It would be predicted that some of these, particularly from smaller families will have *Smad4* mutations since routine colonoscopies that would exclude juvenile polyposis are not a feature of HHT management. In this group, there would be additional clinical screening implications, since for at risk members of juvenile polyposis (JP) families, the British Society of Gastroenterology recommends surveillance colonoscopies and upper gastrointestinal endoscopies, with therapeutic interventions to reduce later risks of colon cancer.¹⁷

The pathogenic mechanisms involved in the development of the HHT vessels are of interest to scientists and clinicians alike. *Endoglin* and *ALK-1* encode proteins expressed predominantly on vascular endothelial cells. Endoglin, *ALK-1*, and the ubiquitously expressed *Smad4* are involved in signalling by the transforming growth factor (TGF)- β superfamily that regulates a diverse series of fundamental pathways in development and pathophysiology. A simplified model of Smad dependent signalling by this superfamily is presented in fig 1, indicating the interactions and functions of the HHT gene products. Ligands signal through heteromeric complexes comprised of type I and type II cell surface receptor serine-threonine kinases.¹⁸ Activated type I receptors phosphorylate cytoplasmic receptor associated Smad proteins (R-Smads). These oligomerise with a co-Smad molecule, *Smad4*, and translocate to the nucleus to act as transcription factors and alter gene expression.

In view of the clinical implications of the new molecular association of HHT with juvenile polyposis, we consider it important to report the linkage analysis in the classical HHT pedigree described by Wallace and Shovlin.⁷ This identifies a

Abbreviations: AVMs, arteriovenous malformations; CM-AVM, capillary malformation-arteriovenous malformation; EBV, Epstein-Barr virus; EC, endothelial cells; HBT, hereditary benign telangiectasia; HHT, hereditary haemorrhagic telangiectasia; HHT1, HHT type 1; HHT2, HHT type 2; JP, juvenile polyposis; JPHT, juvenile polyposis/HHT overlap syndrome; R-Smads, receptor-associated Smad proteins; TGF- β , transforming growth factor- β

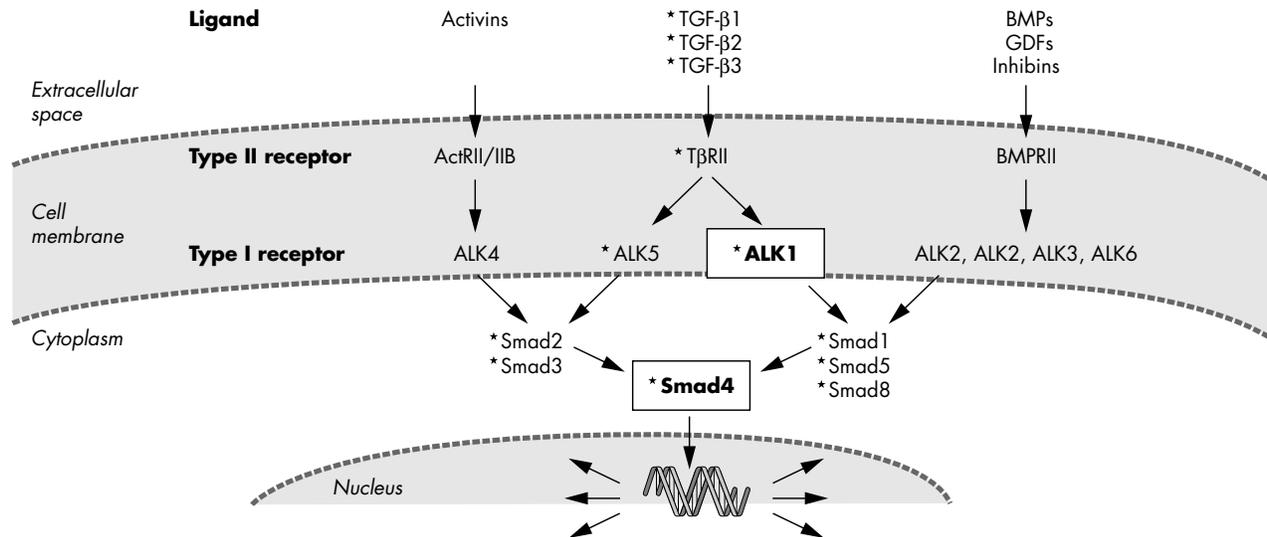


Figure 1 TGF- β superfamily signalling in EC indicating roles of the known HHT gene products. Endoglin associates with all three groups of transmembrane signalling receptors²⁷ and modulates TGF- β 1 induced cellular responses.²⁸ Expression of ALK-1 on EC allows TGF- β 1/T β RII to transduce signals through Smad1 and Smad5, whereas in most cell types, TGF- β /T β RII initiate Smad2 and Smad3 signalling following activation of ALK-5.²⁹ BMPs, bone morphogenetic proteins; GDF, growth/differentiation factors. Boxes: HHT gene products. *: gene excluded by linkage analyses.

new HHT gene locus (*HHT3*) on chromosome 5, resulting in four known types of HHT (table 1).

METHODS

Pedigrees

The proband (fig 2, III.3) was referred to the Hammersmith Hospital for embolization of pulmonary arteriovenous malformations (AVMs). Extended pedigree analysis was performed with informed consent and Multicentre and Local Research Ethics Committee approval (MREC/98/0/42; LREC 99/5637M). The diagnosis of HHT was assigned by the presence of three international consensus diagnostic criteria,¹⁹ that is: affected first degree relative; recurrent, spontaneous nosebleeds; mucocutaneous telangiectasia; and in the case of III.3 and III.4, documented visceral manifestations (pulmonary AVMs). Importantly, telangiectasia were considered diagnostic only if in the correct distribution for HHT (that is, nose, lips, tongue, oral mucosa, finger tips, or ears) and persistent, having developed from late childhood or during adult life. In view of nosebleeds affecting 8–10% of children, with nocturnal nosebleeds a common feature,²⁰ occasional nosebleeds occurring purely in childhood were not considered a diagnostic criterion.

Genotyping and molecular analyses

Genomic DNA was extracted from peripheral venous blood or Isocode mouth swabs (Schleicher and Schuell, Dassel,

Germany) using standard procedures. cDNA was derived from Epstein-Barr virus (EBV) immortalised lymphocyte cell lines which were established on four family members as previously.²¹

A total of 400 fluorescently labelled primer pairs from the ABI Prism Linkage MD-10 Mapping Set (Applied Biosystems, Foster City, CA) were used according to the manufacturer's instructions for a first genome-wide linkage screen. An additional 312 fluorescently labelled polymorphic markers pre-labelled from Applied Biosystems and Research Genetics (Huntsville, AL), or custom synthesised by Sigma-Genosys (Cambridge, UK), were used to fine-map the identified interval, and exclusion map the remainder of the genome. PCR products were size separated on a ABI 7700 capillary sequencer, and analysed using GeneScan software (Applied Biosystems). Candidate genes were analysed by PCR amplification of all exons, exon-intron boundaries, and 40–50 bp of flanking intronic sequence (*endoglin*, *ALK-1*, *Smad4*, *Smad5*), or by sequencing the entire cDNA from EBV immortalised lymphocyte cell lines (*SPARC*). Primer details are available on request.

Two point LOD scores between a putative disease locus and each marker were calculated assuming autosomal dominant inheritance, a disease gene frequency of 0.0001, and equal recombination rates in both sexes. LOD scores were calculated initially with equal allele frequencies. Based on previously published estimates,^{15, 22} for unaffected individuals,

Table 1 HHT genes and pattern of HHT

HHT	HHT features	Inheritance	Comments on HHT	Disease associations	Chromosome	Gene	Phenotype references
HHT1	Classical HHT	AD	Higher frequency of pulmonary AVMs		9	<i>Endoglin</i> ³	Berg <i>et al</i> , ¹³ Shovlin <i>et al</i> ²¹
HHT2	Classical HHT	AD	Milder phenotype	Primary pulmonary hypertension (PPH)	12	<i>ALK-1</i> ⁴	Berg <i>et al</i> , ¹³ Trembath <i>et al</i> ⁴
JPHT	Juvenile polyposis (JP) associated with HHT	AD		Juvenile polyposis	18	<i>Smad4</i> ⁵	Gallione <i>et al</i> ⁶
HHT3	Classical HHT	AD	See text		5	?	Current

AD, autosomal dominant.

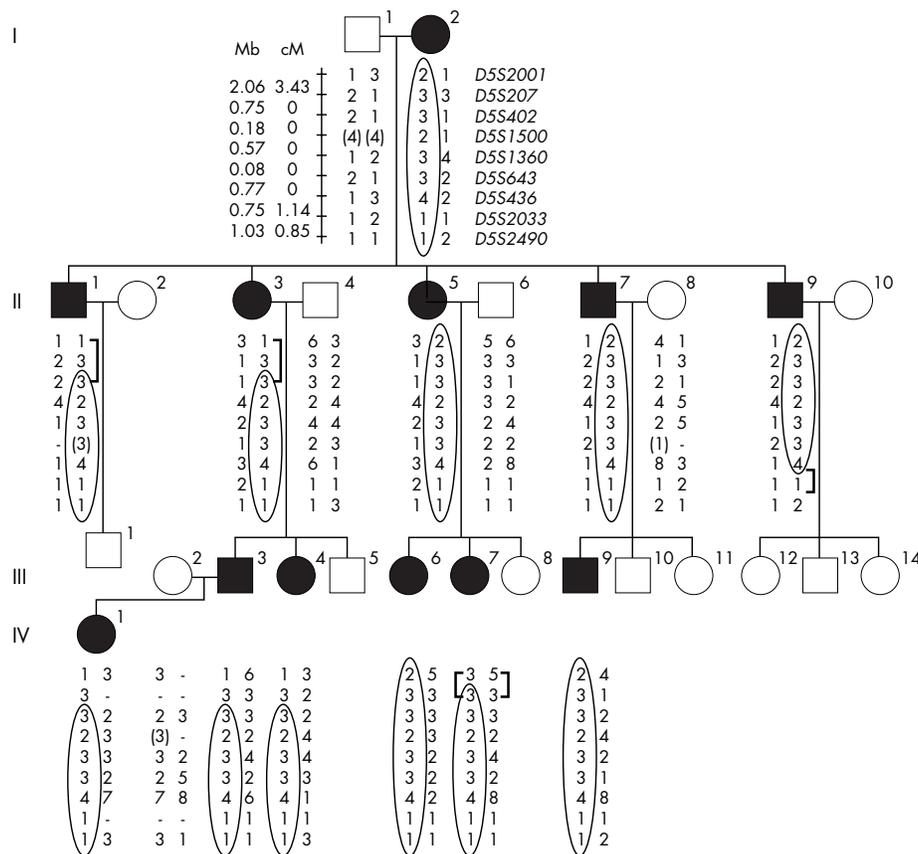


Figure 2 Family pedigree and haplotype analysis. Pedigree: Individuals III.3 and III.4 have pulmonary AVMs. DNA from II.3, II.4, III.3, and III.4 was sequenced for candidate gene analyses. Haplotype analysis: Inheritance of 10 haplotypes in the pedigree indicates segregation of disease haplotype (outlined) in all affected individuals over four generations (II–V). Data on clinically unaffected individuals are not included for ethical reasons, and data on uninformative markers have been excluded for clarity. Key: [or]: recombination events; (): data deduced from offspring; -: data not available.

penetrance was set at $p = 0.8$ between ages 12 and 35 and $p = 0.95$ aged over 35 giving two liability classes. Apparently unaffected children under the age of 12 years were excluded as penetrance is less than 80% at this age.²² SLINK and MLINK analyses²³ were performed using Human Genome Mapping Project (HGMP) computational resources. The order and distances between the markers were derived from Ensembl (<http://www.ensembl.org/>) and GenBank (<http://www.ncbi.nlm.nih.gov/>) databases.

RESULTS

Exclusion of known HHT disease genes

Linkage analyses were performed to confirm the exclusion of *endoglin* and *ALK-1* in the extended pedigree, and to exclude the new JP-HHT gene, *Smad4* (table 2).

Endoglin had been sequenced in full in affected members of the pedigree.²¹ The other two HHT genes were sequenced in four affected family members (III.3, II.3, II.4, III.3, and III.4). No mutations were found in any of the *ALK-1* coding regions or intron-exon boundaries. Although the JP-HHT mutations predominantly occur in the 3' exons of *Smad4*,⁵ all 11 exons and intron-exon boundaries were sequenced. No mutations were found.

Exclusion of core components of the TGF- β signalling pathways

Recognising that the data from the other HHT genes strongly suggested that the disease gene in this pedigree would encode a protein affecting TGF- β signalling, other core components of TGF- β signalling pathways were excluded

by linkage analyses (see fig 1). Literature and database searches revealed that in addition to the proteins illustrated in fig 1, over 100 further proteins are known to interact with the TGF- β superfamily signalling pathways, precluding an exhaustive candidate gene approach. A genome-wide screen was therefore undertaken.

Linkage analyses define the *HHT3* locus on chromosome 5

An initial genome-wide scan excluded 70% of the genome, and identified a 12 cM interval where LOD scores exceeded +2. Information from initial markers was limited due to non-informative meioses. Supplementary adjacent markers were fully informative, generating a two point Z_{\max} of 3.45 at a recombination fraction (θ) of 0.00, and refining the interval. LOD scores were robust to changes in allele frequency (data not shown). The SLINK theoretical Z_{\max} of 4.84 at $\theta = 0.00$ was not achieved due to recombination events in clinically unaffected individuals. The series of two point LOD scores (table 3) and recombination mapping using affected individuals (fig 3) defined the 5.4 cM/6 Mb *HHT3* locus. In this region, all affected family members had inherited a conserved disease associated haplotype (fig 2).

In order to assess the likely frequency of *HHT3*, we studied three families (including two previously unreported) with theoretical $Z_{\max} > 1.6$ in which assignment to *endoglin* or *ALK-1* could not be made. In two, linkage to *HHT3* was excluded. In a third family with a theoretical Z_{\max} of 1.87, a maximum two point LOD score of 1.17 at $\theta = 0.00$ was obtained with *D5S436* on a different disease-segregating haplotype to that

Table 2 Exclusion of known HHT genes

	Mb	cM	$\theta=0.00$	$\theta=0.05$	$\theta=0.10$	$\theta=0.50$
<i>D9S1682</i>	120	132	-9.88	-3.58	-2.14	0.00
<i>Endoglin</i>	124	136				
<i>D9S1825</i>	125	137	-11.38	-4.59	-2.95	0.00
<i>D12S85</i>	46	61	-6.98	-5.99	-4.14	0.00
<i>ALK-1</i>	50	65				
<i>D12S359</i>	52	68	-10.60	-5.83	-4.93	0.00
<i>D18S363</i>	47	71	-22.1	-3.66	-2.30	0.00
<i>Smad4</i>	47	71				
<i>D18S112</i>	52	77	-21.9	-4.06	-2.64	0.00

Haplotype and multipoint data for *endoglin* and *ALK-1* are presented in Wallace and Shovlin.⁷ Mb and cM refer to position on respective chromosome.

illustrated in fig 2. The reduction from the theoretical Z_{max} was due to a single young unaffected recombinant.

Exclusion mapping

To exclude the possibility that an alternative locus had been missed, the remainder of the genome was formally examined. A further 290 polymorphic markers were selected and analysed to ensure that at least two double recombination events would have had to occur in a 2–5 cM interval for a putative locus to have been missed. Highly conservative estimates (excluding genetic interference) based on 500 intervals indicated that the probability of this occurring was between 3.1×10^{-3} and 8×10^{-5} (that is, $p \leq 0.0031$).

Candidate gene analysis

Ensembl identifies 28 genes within the 5.4 cM *HHT3* interval, including 10 of unknown function. Furthermore, the gene for *Smad5*, a strong candidate based on its role in *ALK-1* signalling pathways (fig 1), is assigned on current mapping to within 5 Mb of the *HHT3* interval (fig 3). The *Smad5* gene had been excluded by linkage analyses using markers either side of the published gene locus. However, in view of its strong candidate status due to functional considerations, and the possibility that the precise database positional assignment of *Smad5* was erroneous, all coding exons and flanking intronic sequence were sequenced in affected members of both families. No pathogenic mutations were identified. In addition, the initial 12 cM mapping interval contained *SPARC*, a further attractive candidate gene due to endothelial cell expression and roles in TGF- β 1 mediated proliferative responses.²⁴ *SPARC* cDNA was amplified from EBV immortalised lymphocyte cell lines. The complete transcript was sequenced and no mutations identified.

DISCUSSION

We have identified a novel locus for the autosomal dominant disorder hereditary haemorrhagic telangiectasia (HHT). In the presented family, the disease affects both sexes equally and is indistinguishable from that in other families with HHT. The pulmonary AVM frequency (13%) was not as high as in HHT type I families with *endoglin* mutations, but numbers are too small to suggest that *HHT3* resembles *HHT2* more than *HHT1*. Importantly, no family members have experienced cancer of any form, and none are known to have developed pulmonary hypertension.

Our data do not allow us to address the proportion of HHT families which are due to *HHT3*, as in our four "unassigned" large families, only one categorically maps to chromosome 5. Most HHT families will have mutations in *endoglin* or *ALK-1*, and mutational screening programmes should detect the majority of these. Data from labs employing sensitive quantitative genomic exon PCR screening methods have not detected mutations in as many as 10–15% of classical HHT families (Dr Michelle Letarte, personal communication). In these families, linkage analyses with the chromosome 5 markers should begin to address the likely frequency of *HHT3*.

The *HHT3* locus (5q31.3–5q32) is not the same as that recently identified for hereditary benign telangiectasia (HBT; OMIM 187260) on chromosome 5 (5q14),²⁵ for which the causative gene, *RASA1*, encoding Ras GTPase activating protein 1, has been identified.²⁶ HBT is therefore part of the capillary malformation-arteriovenous malformation (CM-AVM) syndrome,²⁶ and should not be considered a benign allelic variant of HHT as proposed.²⁵ The importance of making this clear distinction is that HHT patients are at significant risk of pulmonary and cerebral AVMs. The diseases can be distinguished clinically by the distinctive skin lesions. In HBT, randomly distributed cutaneous vascular malformations over the head, trunk, and limbs are often congenital (40%) or develop from early childhood. In contrast, HHT telangiectasia have a highly restricted distribution on the mucosa of the nose, lips, oral cavity, conjunctiva, finger tips, ears, and face,¹⁹ develop from teenage years, and become more numerous with age.

As all three identified HHT genes encode proteins involved in TGF- β superfamily signalling, we anticipate that the disease gene responsible for *HHT3* will also encode a protein involved in Smad dependent TGF- β signalling. In keeping with the expression patterns of *endoglin* and *ALK-1* which are transmembrane proteins predominantly expressed on vascular endothelial cells (EC), we predict that the disease gene for this "pure" form of HHT will also display EC restricted expression. Identification of this gene will be

Table 3 LOD scores (Z) spanning the *HHT3* interval

Marker	cM	Mb	0	0.01	0.05	0.1	0.2	0.3	0.4	0.5
<i>D5S2011</i>	144.06	141.25	-8.82	-2.55	-0.58	0.15	0.64	0.67	0.44	0.00
<i>D5S2017</i>	145.21	141.76	-3.76	0.19	0.79	0.95	0.91	0.68	0.36	0.00
<i>D5S1972</i>	146.73	142.37	1.44	1.43	1.39	1.31	1.07	0.72	0.32	0.00
<i>D5S207</i>	147.49	143.31	1.72	1.71	1.67	1.59	1.35	1.01	0.56	0.00
<i>D5S402</i>	147.49	144.06	2.42	2.40	2.30	2.15	1.74	1.21	0.58	0.00
<i>D5S1480</i>	147.49	144.17	1.72	1.71	1.67	1.59	1.35	1.01	0.56	0.00
<i>D5S1500</i>	147.49	144.24	3.10	3.06	2.88	2.64	2.09	1.43	0.69	0.00
<i>D5S210</i>	147.49	144.49	2.24	2.23	2.16	2.03	1.70	1.25	0.69	0.00
<i>D5S1360</i>	147.49	144.92	3.00	2.97	2.84	2.64	2.13	1.49	0.74	0.00
<i>D5S643</i>	147.49	145.00	3.45	3.41	3.25	3.01	2.42	1.69	0.84	0.00
<i>D5S436</i>	147.49	145.23	3.45	3.41	3.25	3.01	2.42	1.69	0.84	0.00
<i>D5S2033</i>	148.63	145.98	0.40	0.46	0.59	0.65	0.62	0.49	0.28	0.00
<i>D5S2847</i>	149.48	147.00	-2.60	1.34	1.83	1.87	1.59	1.14	0.59	0.00
<i>D5S2490</i>	149.48	147.01	-4.12	-0.16	0.43	0.58	0.57	0.40	0.19	0.00
<i>D5S670</i>	156.47	152.88	-3.90	-1.90	-0.55	-0.03	0.34	0.38	0.25	0.00

Marker positions ranked according to the Marshfield map.

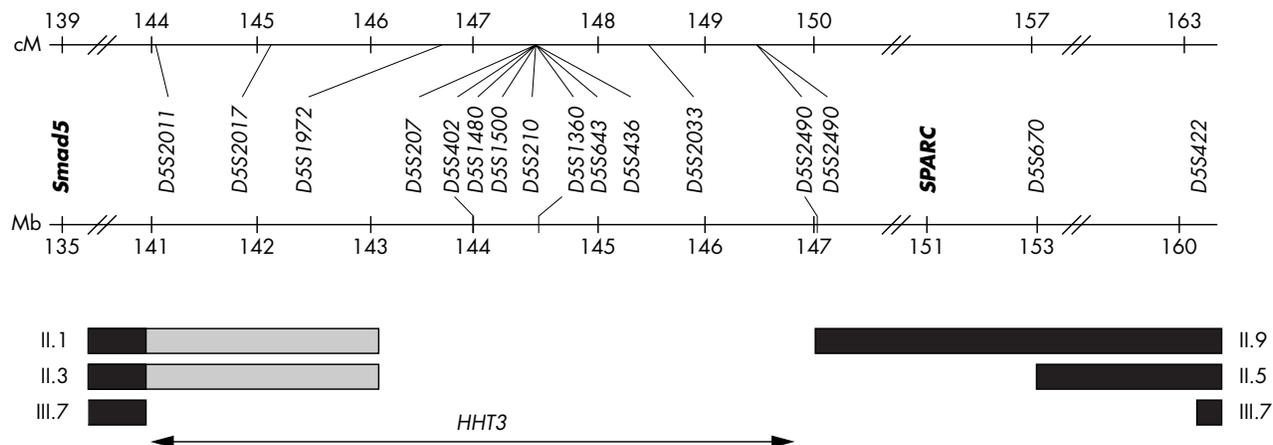


Figure 3 Recombination mapping of interval using affected family members. Pedigree numbers as in fig 2. Black bars indicate definite recombination events; shaded bars indicate uninformative markers. The locations of the candidate genes *Smad5* and *SPARC* (but not *RASA1* at 86.6 Mb) are illustrated.

important not only for clinical diagnostic services but also to elucidate the mechanisms of TGF- β superfamily signalling in vascular endothelium.

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ELECTRONIC-DATABASE INFORMATION



The Ensembl website can be found at <http://www.ensembl.org/> and GenBank at <http://www.ncbi.nlm.nih.gov/>.

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CORRECTION

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It has come to our attention that in figure 3 of BE Baysal *et al* (*J Med Genet* 2004;**41**:703-9) that some of the lines were displaced. Below is a corrected figure. The journal apologises for this error.

