LETTER TO JMG

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

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H ereditary 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. 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 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 Baysal and the more recent additions of multiple mutations in SDHB and SDHD. All reported mutations are single nucleotide alterations leading to splice site, missense, nonsense, or frameshift mutations, or intronic deletions and insertions of up to four nucleotides, which have been detected through exon 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. However, analyses of SDHC in four series of patients with paraganglioma or pheochromocytoma 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, suggesting operation of genomic imprinting on SDHD. In contrast, SDHB mutations are transmitted both paternally and maternally. Thus far, transmissions of SDHC mutations causing disease occurred through mothers in the one multiplex family and in one isolated case. 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 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 techniques 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

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 materially, 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 imprinting transmission in hereditary paraganglioma is restricted to SDHD among complex II genes.

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'-TTGGATGGCCCTGGGCT-3' and 120R: 5'-AACAAGCATAGC TCTCAAGGT-3' (512 bp); the amplicon is located ~5 kb downstream of exon 6; (2) 123F: 5'-TGTGTCCTCAGTTGGAT GCC-3' and 123R: 5'-GAAGATTCTGGAAGGAGAC-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'-GAACAATTGATTTGGATAG-3' and 124R: 5'-CCATG TTAAACCTACGTTAAC-3' (236 bp); the amplicon is located ~8.5 kb downstream of exon 6; (2) 110F: 5'-GGAGAAAAATATATGTTTTTTAATGAAG-3' and 110R: 5'-GAGATTCTCAGAAATCCTT-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'-CCTTTAGAATACTAGTTCTGA-3' and 124R: 5'-CCATGTTTAACCTACAGCTT-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

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**Figure 1** A simplified pedigree of the multiplex family with SDHC deletion. Differently patterned long vertical bars symbolize 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) [D1S2635 and D1S2844] and the location of the deletion identified in this study is shown on the right. D1S2844 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.
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 = (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 × [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 ≈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](Image)

**Table 1** Phenotypic characteristics of affected and carrier subjects

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

*Information obtained through medical history and interview.

**SDHC**

Letter to JMG 705

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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. 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 being 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, 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. 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...
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. The adjoining of two similar AluY elements by the deletion and the identification of a 4 bp insertion at the 4-bp insertion at the

<table>
<thead>
<tr>
<th>Locus/repeat motif</th>
<th>Location</th>
<th>Primer sequences (5' to 3')</th>
<th>Allele no/range (bp)</th>
<th>Expected heterozygosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDHC-CA-2/(Ca)n</td>
<td>3 kb upstream of exon 1</td>
<td>GCAAAAGCAAGGACAAAGAGGT TATATCCAACACTTCAGAAACCT</td>
<td>10/127–147</td>
<td>0.85</td>
</tr>
<tr>
<td>SDHC-CA-3/(Ca)n</td>
<td>Intron 4</td>
<td>TTATGGGAAACAACTGGCCTCT GCAGGAAGCTGAGATCGCA</td>
<td>4/190–211</td>
<td>0.66</td>
</tr>
<tr>
<td>SDHC-3'-STRP/complex (TA)n(GA)n</td>
<td>33.5 kb downstream of exon 6</td>
<td>CAGTGAGCCGAGATCAATGG TGCACCAACAGGGCCTGCT</td>
<td>6/150–160</td>
<td>0.72</td>
</tr>
<tr>
<td>SDHC-Tetra/(GAAA)n</td>
<td>68 kb downstream of exon 6</td>
<td>ACTCCAGTCTGGGCTGGAGCA TGAGTTTCCTGGATTCTACAGA</td>
<td>6/195–219</td>
<td>0.79</td>
</tr>
</tbody>
</table>
jungtion suggests that a homologous Alu-Alu recombination between two highly similar elements, that occurred intrachromosomally 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.\(^9\)

Availability of paraffinised 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 Muller\(^6\) and suggest that SDHC 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,\(^1\) 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 prepair filler DNA insertion at the junction. To further assess the possibility of a common origin, we genotyped three pre

REFERENCES

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. 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 (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 evaluation of pulmonary arteriovenous malformations (AVMs). 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.

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 evaluation 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.

HHT is a disease with late onset penetrance (>90% by 40 years; 97% by 60 years), genetic screening programmes have been introduced. 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.

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. 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 Showlin. This identifies a novel gene, Smad4, as a candidate gene for HHT.

Abbreviations: AVMs, arteriovenous malformations; CM-AVM, capillary malformation-arteriovenous malformation; EBV, Ebstein-Barr virus; EC, endothelial cells; HBT, hereditary benign telangiectasia; HHT, hereditary haemorrhagic telangiectasia; HHT1, HHT type 1; HHT2, HHT type 2; JP, juvenile polyposis; JHHT, juvenile polyposis/HHT overlap syndrome; R-Smads, receptor-associated Smad proteins; TGF-β, transforming growth factor-β
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, 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, 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 Ebstein-Barr virus (EBV) immortalised lymphocyte cell lines which were established on four family members as previously.

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.

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, for unaffected individuals,
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 unaffected children under the age of 12 years were excluded giving two liability classes. Apparently 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.

### 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. 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, all 11 exons and intron-exon boundaries were sequenced. No mutations were found.

#### Exclusion of core components of the TGF-\( \beta \) 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-\( \beta \) signalling, other core components of TGF-\( \beta \) 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-\( \beta \) 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 \( Z_{\text{max}} \) of 3.45 at a recombination fraction \( \theta = 0.00 \), and refining the interval. LOD scores were robust to changes in allele frequency (data not shown). The SLINK theoretical \( Z_{\text{max}} \) 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 \( Z_{\text{max}} > 1.6 \) 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 \( Z_{\text{max}} \) 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
illustrated in fig 2. The reduction from the theoretical $Z_{\text{max}}$ 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 $3.1 \times 10^{-3}$ and $8 \times 10^{-5}$ (that is, $p < 0.0031$).

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 mediated proliferative responses. 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 1 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 (5q13.1–5q32) is not the same as that recently identified for hereditary benign telangiectasia (HBT; OMIM 187260) on chromosome 5 (5q14), for which the causative gene, RASA1, encoding Ras GTPase activating protein 1, has been identified. HBT is therefore part of the capillary malformation-arteriovenous malformation (CM-AVM) syndrome, and should not be considered a benign allelic variant of HHT as proposed. 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, 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

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**Table 2** Exclusion of known HHT genes

<table>
<thead>
<tr>
<th>Marker</th>
<th>cM</th>
<th>Mb</th>
<th>$\theta = 0.00$</th>
<th>$\theta = 0.05$</th>
<th>$\theta = 0.10$</th>
<th>$\theta = 0.50$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D9S1682</td>
<td>120</td>
<td>132</td>
<td>-9.88</td>
<td>-3.58</td>
<td>-2.14</td>
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</tr>
<tr>
<td>Endoglin</td>
<td>124</td>
<td>136</td>
<td>46</td>
<td>61</td>
<td>-6.98</td>
<td>-5.99</td>
</tr>
<tr>
<td>D9S1825</td>
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<td>137</td>
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<td>-4.59</td>
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</tr>
<tr>
<td>D12S59</td>
<td>47</td>
<td>71</td>
<td>-22.1</td>
<td>-3.66</td>
<td>-2.30</td>
<td>0.00</td>
</tr>
<tr>
<td>Smad4</td>
<td>47</td>
<td>71</td>
<td>-21.9</td>
<td>-4.06</td>
<td>-2.64</td>
<td>0.00</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Marker</th>
<th>cM</th>
<th>Mb</th>
<th>0</th>
<th>0.01</th>
<th>0.05</th>
<th>0.1</th>
<th>0.2</th>
<th>0.3</th>
<th>0.4</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSS2011</td>
<td>144.06</td>
<td>141.25</td>
<td>-8.82</td>
<td>-2.55</td>
<td>-0.58</td>
<td>0.15</td>
<td>0.64</td>
<td>0.67</td>
<td>0.44</td>
<td>0.00</td>
</tr>
<tr>
<td>DSS2017</td>
<td>145.21</td>
<td>141.76</td>
<td>-3.76</td>
<td>0.19</td>
<td>0.79</td>
<td>0.95</td>
<td>0.91</td>
<td>0.68</td>
<td>0.36</td>
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<td>146.73</td>
<td>142.37</td>
<td>1.44</td>
<td>1.43</td>
<td>1.39</td>
<td>1.31</td>
<td>1.07</td>
<td>0.72</td>
<td>0.32</td>
<td>0.00</td>
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<tr>
<td>DSS2007</td>
<td>147.49</td>
<td>143.31</td>
<td>1.72</td>
<td>1.71</td>
<td>1.67</td>
<td>1.59</td>
<td>1.35</td>
<td>1.01</td>
<td>0.56</td>
<td>0.00</td>
</tr>
<tr>
<td>DSS2002</td>
<td>147.49</td>
<td>144.06</td>
<td>2.42</td>
<td>2.40</td>
<td>2.30</td>
<td>2.15</td>
<td>1.74</td>
<td>1.21</td>
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<td>DSS1480</td>
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<td>144.17</td>
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<td>1.71</td>
<td>1.67</td>
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<td>DSS2210</td>
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<td>144.49</td>
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<td>DSS3600</td>
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<td>144.92</td>
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<td>2.97</td>
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<td>3.41</td>
<td>3.25</td>
<td>3.01</td>
<td>2.42</td>
<td>1.69</td>
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</tr>
<tr>
<td>DSS2033</td>
<td>148.63</td>
<td>145.98</td>
<td>0.40</td>
<td>0.46</td>
<td>0.59</td>
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<td>147.00</td>
<td>-2.60</td>
<td>1.34</td>
<td>1.83</td>
<td>1.87</td>
<td>1.59</td>
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</tr>
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<td>-0.55</td>
<td>-0.03</td>
<td>0.34</td>
<td>0.38</td>
<td>0.25</td>
<td>0.00</td>
</tr>
</tbody>
</table>

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

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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.

ACKNOWLEDGEMENTS

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ELECTRONIC-DATABASE INFORMATION


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Competing interests: none declared

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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.

Chromatogram of the deletion junction