

Endoglin, a TGF- β binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1

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Hereditary haemorrhagic telangiectasia (HHT) is an autosomal dominant disorder characterized by multisystemic vascular dysplasia and recurrent haemorrhage. Linkage for some families has been established to chromosome 9q33–q34. In the present study, endoglin, a transforming growth factor β (TGF- β) binding protein, was analysed as a candidate gene for the disorder based on chromosomal location, expression pattern and function. We have identified mutations in three affected individuals: a C to G substitution converting a tyrosine to a termination codon, a 39 base pair deletion and a 2 basepair deletion which creates a premature termination codon. We have identified endoglin as the HHT gene mapping to 9q3 and have established HHT as the first human disease defined by a mutation in a member of the TGF- β receptor complex.

Hereditary haemorrhagic telangiectasia (HHT) or Osler-Weber-Rendu disease (OMIM #18730) is an autosomal dominant disorder characterized by multisystemic vascular dysplasia and recurrent haemorrhage. The disorder is named after the recurrent haemorrhage from vascular lesions, especially in the nasal mucosa and gastrointestinal tract, and for the presence of mucosal, dermal and visceral telangiectases. Pulmonary arteriovenous malformations (PAVMs) occur in approximately 20% of patients and are associated with serious complications including stroke and brain abscess. Other neurological manifestations include cerebral arteriovenous malformation, aneurysm and migraine headache.

Ultrastructural analyses of the vascular dysplasia seen in affected individuals have failed to demonstrate a unique pathological abnormality that might suggest the nature of the primary biochemical defect. Studies indicate that the dilated channels of telangiectases are lined by a single layer of endothelium attached to a continuous basement membrane^{1,2}. The earliest event in the formation of telangiectases appears to be dilation of post-capillary venules³. Eventually the dilated venules connect to enlarging arterioles through capillary segments which later disappear, creating direct arteriolar–venular connections. This sequence of events is associated with a perivascular mononuclear infiltrate³. Various explanations have been put forward to explain the angiodysplasia seen in HHT including endothelial cell degeneration⁴, defects in endothelial junctions², lack of elastic fibres and incomplete smooth muscle cell coating of the vessels¹, and weak connective tissue surrounding the vessel⁴.

Genetic linkage for some HHT families was recently established to markers on chromosome 9q33–q34 (refs 5,6), and the locus was named *OWR1*. Genetic heterogeneity was established with the identification of some families clearly not linked to this region^{6–9}. However, a predisposition to PAVMs in multiple affected members of a family correlates with 9q3 linkage^{7–9}, establishing a valuable diagnostic criterion for *OWR1*. The identification of key obligate recombinants in affected individuals allowed refinement of the *OWR1* locus and placed the most likely candidate interval between *D9S60* and *D9S61* in a 2 centiMorgan (cM) interval^{6,7,9}. The *COL5A1* gene mapping to chromosome 9q34 has been considered a candidate gene for this disorder, but our mapping studies indicate it lies distal to the candidate interval with obligate recombinants identified in *OWR1* kindreds¹⁰.

Here, we investigate a strong candidate for the *OWR1* disease gene. Endoglin is a homodimeric integral membrane glycoprotein expressed at high levels on human vascular endothelial cells of capillaries, arterioles and venules in all tissues examined¹¹. On endothelial cells, endoglin is the most abundant transforming growth factor β (TGF- β) binding protein¹². In the presence of TGF- β ligand, endoglin can associate with the signaling receptors RI and RII and is thought to initiate response to the growth factor¹³ (Letarte *et al.*, unpublished observations). TGF- β is the prototype of a family of at least 25 growth factors which regulate growth, differentiation, motility, tissue remodeling, wound repair and programmed cell death in many cell types¹⁴.

Endoglin has been mapped to human chromosome 9q34 using fluorescence *in situ* hybridization (FISH)¹⁵. It lies in a broad region on 9q33–q34 that shows conserved

Exon 1 ▼

1 ATGGACCGCGGCACGCTCCCTCTGGCTGTGCGCTGCTGGCCAGCTGCAGCCTCAGCCCCACAAGTCTTGCAAAACAGTCCATTGT
1 MetAspArgGlyThrLeuProLeuAlaValAlaLeuLeuLeuAlaSerCysSerLeuSerProThrSerLeuAlaGluThrValHisCys

Exon 2

91 GACCTTCAGCCTGTGGGCCCCGAGAGGGGCGAGGTGACATATACCCTAGCCAGGTCTCGAAGGGCTGCGTGGCTCAGCCCCCAATGCC
31 AspLeuGlnProValGlyProGluArgGlyGluValThrTyrThrThrSerGlnValSerLysGlyCysValAlaGlnAlaProAsnAla

▼

181 ATCTTGAAGTCCATGCTCTCTCTGGAGTCCCAACGGGCCCCGCACAGCTGGAGCTGACTCTCCAGGCATCCAAGCAAAATGGCACC
61 IleLeuGluValHisValLeuPheLeuGluPheProThrGlyProSerGlnLeuGluLeuThrLeuGlnAlaSerLysGln **AsnGlyThr**

Exon 3 ▼

271 TGGCCCCGAGAGGTCTCTGGTCCCTCAGTGTAACAGCAGTGTCTCTCGCATCTCCAGGCCCTGGGAATCCCACTGCACTTGGCCTAC
91 TrpProArgGluValLeuLeuValLeuSerValAsnSerSerValPheLeuHisLeuGlnAlaLeuGlyIleProLeuHisLeuAlaTyr

Exon 4

361 AATTCAGCCTGGTCACCTTCCAAGAGCCCCGGGGGTCAACACCACAGAGCTGCCATCCTTCCCCAAGACCCAGATCCTTGAGTGGGCA
121 **AsnSerSer**LeuValThrPheGlnGluProProGlyVal **AsnThrThr**GluLeuProSerPheProLysThrGlnIleLeuGluTrpAla

▼

451 GCTGAGAGGGGCCCCATCACCTCTGCTGCTGAGTGAATGACCCCCAGAGCATCCTCTCCGACTGGGCCAAGCCAGGGGTCACTGTCC
151 AlaGluArgGlyProIleThrSerAlaAlaGluLeuAsnAspProGlnSerIleLeuLeuArgLeuGlyGlnAlaGlnGlySerLeuSer

Exon 5

541 TTCTGCATGCTGGAAGCCAGCCAGGACATGGGCGCAGCCTCGAGTGGCGGCGCTACTCCAGCCTTGGTCCGGGGTGCCTACTGGAA
181 PheCysMetLeuGluAlaSerGlnAspMetGlyArgThrLeuGluTrpArgProArgThrProAlaLeuValArgGlyCysHisLeuGlu

▼

631 GCGGTGGCCGGCCACAAGGAGGCGCACATCTCTGAGGGTCTGCGGGCCACTCGGCCGGGCCCCGACGGTGACGGTGAAGGTGGAAGT
211 GlyValAlaGlyHisLysGluAlaHisIleLeuArgValLeuProGlyHisSerAlaGlyProArgThrValThrValLysValGluLeu

Exon 6

721 AGCTGCGCACCCGGGATCTCGATGCCGCTCATCTGCAGGGTCCCCCTACGTGTCTCTGGCTCATCGACGCCAACCCACAACATCGAC
241 SerCysAlaProGlyAspLeuAspAlaValLeuIleLeuGlnGlyProProTyrValSerTrpLeuIleAspAlaAsnHisAsnMetGln

▼ * ▼

811 ATCTGGACCACCTGGAGAATACTCCTCAAGATCTTCCAGAGAAAAACATTCGTGGCTTCAAGCTCCAGACACACCTCAAGCCCTCTG
271 IleTrpThrThrGlyGluTyrSerPheLysIlePheProGluLysAsnIleArgGlyPheLysLeuProAspThrProGlnGlyLeuLeu

Exon 7

901 GGGGAGGCCCGGATGCTCAATGCCAGCATGTGGCATCCTTCGTGGAGCTACCGCTGGCCAGCATGTCTCACTTCATGCCCTCCAGTGC
301 GlyGluAlaArgMetLeu **AsnAlaSer**IleValAlaSerPheValGluLeuProLeuAlaSerIleValSerLeuHisAlaSerSerCys

▼

Exon 8

991 GGTGGTAGGCTCAGACCTCACCCGCACCGATCCAGACCCTCTCCCAAGGACACTGTAGCCCCGAGCTGCTCATGTCTTGATCCAG
331 GlyGlyArgLeuGlnThrSerProAlaProIleGlnThrThrProProLysAspThrCysSerProGluLeuLeuMetSerLeuIleGln

▼

1081 ACAAAAGTGTGCCGACGACGCCATGACCCCTGGTACTAAAGAAAGAGCTTGTTCGCGCATTTGAAGTGCACCATCACGGGCTGACCTTCTGG
361 ThrLysCysAlaAspAspAlaMetThrLeuValLeuLysLysGluLeuValAlaHisLeuLysCysThrIleThrGlyLeuThrPheTrp

Exon 9

1171 GACCCAGCTGTGAGGCAGAGGACAGGGGTGACAAGTTGTCTTGCAGTGTCTACTCCAGCTGTGGCATGCAGGTGTCAGCAAGTATG
391 AspProSerCysGluAlaGluAspArgGlyAspLysPheValLeuArgSerAlaTyrSerSerCysGlyMetGlnValSerAlaSerMet

▼

1261 ATCAGCAATGAGGCGGTGGTCAATATCTGTGCGAGCTCATCACCACAGCGAAAAAGGTGCACTGCCTCAACATGGACAGCCTCTCTTTC
421 IleSerAsnGluAlaValValAsnIleLeuSerSerSerSerProGlnArgLysLysValHisCysLeuAsnMetAspSerLeuSerPhe

Exon 10 ▼

1351 CAGCTGGGCTCTACCTCAGCCACACTTCTCCAGGCTCCAACACCATCGAGCCGGGCGAGCAGAGCTTTGTGCAGGTCCAGAGTGTCC
451 GlnLeuGlyLeuTyrLeuSerProHisPheLeuGlnAlaSerAsnThrIleGluProGlyGlnGlnSerPheValGlnValArgValSer

1441 CCATCCGCTCTCCGAGTTCCTGCTCAGTGTAGACAGCTGCCACCTGGACTTGGGGCTGAGGGAGGCACCGTGAAGTCCATCCAGGGCCGG
481 ProSerValSerGluPheLeuLeuGlnLeuAspSerCysHisLeuAspLeuGlyProGluGlyGlyThrValGluLeuIleGlnGlyArg

Exon 11

1531 GCGGCCAAGGGCAACTGTGTGAGCTGCTGTCCCAAGCCCGAGGGTGAACCCGCGCTTCAGCTTCTCTCCACTTCTACACAGTACCC
511 AlaAlaLysGlyAsnCysValSerLeuLeuSerProSerProGluGlyAspProArgPheSerPheLeuLeuHisPheTyrThrValPro

▼

1621 ATACCCAAAACCGGCACCCCTCAGCTGCACGGTAGCCCTGCGTCCCAAGACCGGGTCTCAAGACCAGGAAGTCCATAGGACTGTCTTCATG
541 IleProLysThrGlyThrLeuSerCysThrValAlaLeuArgProLysThrGlySerGlnAspGlnGluValHisArgThrValPheMet

Exon 12 ▼ **Exon 13**

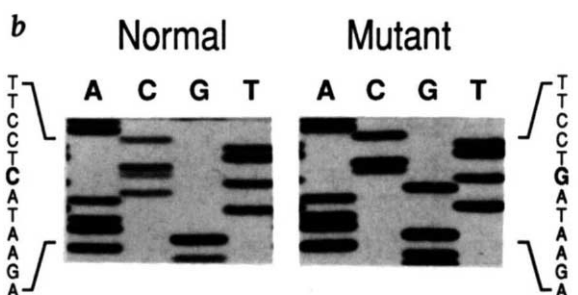
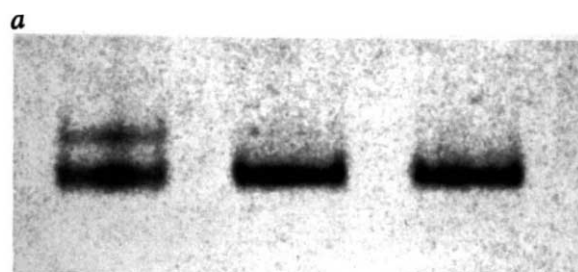
1711 CGCTTGAACATCATCAGCCCTGACCTGTCTGGTTCACAAGCAAAGGCCCTCGTCTGCCCGCGTCTGGGCATCACCTTTGGTGCCTTC
571 ArgLeuAsnIleIleSerProAspLeuSerGlyCysThrSerLysGlyLeuValLeuProAlaValLeuGlyIleThrPheGlyAlaPhe

▼

1801 CTCATCGGGCCCTGCTCACTGCTGCACTCTGGTACATCTACTCGCACAGCGTTCCCCAGCAAAGCGGGAGCCCGTGGTGGCGGTGGCT
601 LeuIleGlyAlaLeuLeuThrAlaAlaLeuTrpTyrIleTyrSerHisThrArgSerProSerLysArgGluProValValAlaValAla

Exon 14

1891 GCCCGGCTCTCGGAGAGCAGCACCAACCACAGCATCGGGAGCACCCAGAGCACCCCTGCTCCACCAGCAGCATGGCATAGCCC
631 AlaProAlaSerSerGluSerSerSerThrAsnHisSerIleGlySerThrGlnSerThrProCysSerThrSerSerMetAla***
CGGCCCCCGGCTCGCCAGCAGGAGACTGAGCAGCCGCGCAGCTGGGAGCACTGGTGTGAAGTCAACCTGGGAGCCAGTCTCCACT
CGACCCAGAATGGAGCTGCTCTCCGCGCTACCCCTCCCGCTCCCTCTCAGAGGCTGCTGCCAGTGCAGCCACTGGCTTGGAAACCC
TTGGGGTCCCTCCACCCACAGAACCTTCAACCCAGTGGGTCTGGGATATGGCTGCCAGGAGACAGACCCTTCCACCGCTGTGTGAAA
AACCCAAGTCCCTGTCAATTGAACCTGGATC



	820	
Normal	ACT GGA GAA <u>TAC</u> TCC TTC AAG	
	Thr Gly Glu Tyr Ser Phe Lys	
Mutant	ACT GGA GAA <u>TAG</u> TCC TTC AAG	
	Thr Gly Glu ***	

synteny with mouse chromosome 2. The mouse endoglin (*End* or *Eng*) locus is genetically inseparable with the genetic marker adenylate kinase 1, *AK1*¹⁶. Human *AK1* is itself genetically inseparable from *D9S60*¹⁷, which forms the proximal border of the candidate region for *OWRI* (refs 6,7,9). FISH using interphase nuclei places *AK1* between *D9S60* and *D9S61* on human chromosome 9q¹⁸, within the smallest candidate interval. We inferred that the tightly linked human endoglin gene would also lie within this interval and be a candidate gene for *OWRI*.

Genomic structure of endoglin

As an initial screen for gross abnormalities in the endoglin gene in affected HHT individuals, Southern blots of DNA from the probands of 33 unrelated families were probed with a nearly complete cDNA of endoglin, clone 18A¹¹. This analysis using three restriction endonucleases revealed no gross abnormalities of the endoglin gene in these samples. RT-PCR was attempted using RNA prepared from several EBV-transformed lymphoblast lines established from our patient cohort, but expression levels of endoglin appeared to be too low to allow routine amplification in a single round (35 cycles) of PCR. As the

Fig. 2 Point mutation in sample 1159. **a**, Heteroduplex analysis showing a shift in an affected proband (sample 1159; lane 1) next to two samples (lanes 2 and 3) not displaying this anomaly. **b**, Sequence of representative clones revealing the normal (C) and mutant sequence (G) of amplified exon 7 in sample 1159. **c**, Consequence of the substitution in sample 1159. This C to G substitution converts a tyrosine to a stop codon at amino acid 277.

expression of endoglin is restricted to endothelial cells, activated monocytes¹⁹, syncytiotrophoblasts²⁰, and certain stromal cells²¹, screening for mutations within endoglin cDNA was not feasible. We therefore began to determine the genomic structure of endoglin.

A gridded cosmid chromosome 9 library was screened with the 18A cDNA probe and 17 cosmids were obtained. Southern analysis of these clones in comparison with total genomic DNA revealed that one cosmid, 21c10, appeared to contain most of the gene. This cosmid was subcloned into a phagemid library which was screened for positive plaques with the 18A cDNA probe. Hybridizing clones were converted to plasmids and sequenced using vector primers flanking the cloning site to identify intron-exon borders.

Preliminary sequence analysis suggests that the coding region of endoglin contains 14 exons (Fig. 1). One or more splice junctions may remain unidentified within the 5' end of the gene, as the sequence denoted exon 1, which contains the putative signal peptide, was found to be missing in the 21c10 cosmid. There is also evidence for alternative splicing variants of the endoglin transcript (Pichantes *et al.*, unpublished observations). Since only one variant was used to identify subclones for genomic sequencing, it is possible that additional exons exist within the depicted coding region (Fig. 1). (The exon number assignments must be regarded as preliminary until the entire gene structure is resolved.)

The 14 exons are sufficiently small to allow for PCR amplification of each as a single unit (Table 1). The smallest is exon 12 which contains the complete membrane spanning domain and is 55 basepairs (bp) in length. The longest exon completely contained within the coding region is exon 11 (258 bp). Exon 14 contains at least 429 bp but contains only 125 bases of coding information, the remainder being the 3' untranslated region.

Identification of HHT mutations

In an initial screen for mutations, primers located within the introns flanking exons 7 and 11 (the first exons to be identified) were designed to establish PCR assays for each exon (see Methodology). A panel of 68 DNA samples was used for the mutation screen. These were collected from probands of unrelated families, most of which were members of kindreds with PAVM involvement, increasing

◀ Fig. 1 Genomic structure of endoglin. The cDNA sequence of endoglin is shown with the amino acid sequence below. The nucleotide and amino acid positions are based on numbering the A in the ATG start codon of the full-length L-form of endoglin³⁶ as nucleotide number 1. Exons 1 through 14 are labelled above the cDNA sequence in bold and the intron/exon borders are marked with arrows. Exon 7 codes for amino acids 273–331; exon 11 spans residues 477–562. The four potential N-linked glycosylation sites are in boldface, italicized type and are underlined. The membrane spanning domain is double underlined. The positions of the mutations described in this report are shown in relation to the gene structure; the C to G change at nucleotide 831 is indicated by a star and the positions of the two deletions are underlined (nucleotides 882–920 and 1553–1554). The 2 bp deletion creates a premature termination codon which is indicated by bold type.

Table 1 PCR assays for endoglin exons

Exon	Size	Forward primer Sequence (5'-3')	Reverse primer Sequence (5'-3')	PCR prod. size	Buffer	[MgCl ₂] (mM)	Annealing temp. (°C)	No. cycles
1	> 67							
2	152			413	BMB	1.5	60	35
3	141							
4	163	ttctgacacctctacatgg	ctcttggtgcccaagttt	330	TNK 50	1.0	51	30
5	166	gggctctgttaggtgcag	gggtggggcttataaggga	294	TNK 25	1.0	57	35
6	127	ctgtccgcttcaggttccatc	ggaaacttcctgatccagaggtt	230	TNK 100	1.5	59	40
7	176	gaggcctggcataaccct	gtggccactgatccaagg	315	BMB	1.5	60	35
8	141							
9	178							
10	117	agattgaccaagtctccctccc	aggctgtctccctcctgactct	227	TNK 50	1.0	61	35
11	258	actcaggggtgggaactctt	ccttccatgcaaaccacag	430	TNK 50	1.0	57	32
12	55	gagtaaacctggaagccgc	gccactagaacaaaccgcag	164	TNK 100	1.0	55	35
13	111	ccagcacaacagggtaggggat	ctcagaggcttcactgggctcc	255	TNK 50	1.0	61	35
14	> 429	tgaagcctctgagggattgagg	gagttcacaccagtgtcccag	267	TNK 50	1.0	57	40

the likelihood that the individuals would harbour mutations at the *OWR1* locus^{7,9}. Included in our analysis was one member from each of eight 9q3-linked families previously described^{5,7,9}. Heteroduplex analysis was performed on amplified products from this cohort as a screen for potential mutations. Abnormal PCR products seen on these gels were directly sequenced for further analysis.

With this initial screen, we have identified three mutations in affected individuals. The first mutation was identified by a heteroduplex shift in the exon 7 PCR product from sample 1159 (Fig. 2a). The products of two independent PCR reactions were directly sequenced and a C (normal) and a G (mutation) at nucleotide position 831 were clearly visible. PCR products amplified from this individual were then cloned and individual clones sequenced to validate the results of the direct sequencing (Fig. 2b). This change converts a tyrosine at codon 277 to a termination codon (Fig. 2c). This mutation is present in the proband of a pedigree with multiple affected members with documented PAVMs. However, additional members of this family were not available for analysis. The truncated protein resulting from this mutation would comprise only half of the extracellular domain and lack the membrane spanning and cytoplasmic domains.

Amplification of exon 7 in sample 8019 revealed a second mutation in a family (Family 3186) previously linked to 9q3 (ref. 9). A second PCR fragment smaller than the wild-type fragment was visible in both agarose gels and heteroduplex analysis, suggesting the existence of a deletion. The smaller fragment was not seen in 278 normal chromosomes and is unlikely to be a polymorphism. Sequence analysis of the PCR products revealed a 39 bp deletion in the exon beginning at nucleotide position 882 of endoglin (Fig. 3a). This in-frame deletion removes 13 amino acids (amino acids 295 to 307) and alters the first amino acid of a potential N-linked glycosylation site (see Fig. 1). Amplification of this exon revealed the presence of the deletion in all affected family members, but no unaffected members (Fig. 3b).

Heteroduplex analysis of amplified exon 11 revealed a very pronounced band in sample 2061 that was not visible with agarose gel electrophoresis (Fig. 4a). Independent clones of the PCR product were sequenced and revealed the wild-type sequence and a 2 bp deletion beginning with nucleotide 1553 of endoglin (Fig. 4b). This deletion creates a *MaeIII* restriction site. This sample was from the proband of a family with multiple affected members displaying PAVMs. Exon 11 was amplified from all available family members and digested with *MaeIII*. All affected family members share the additional *MaeIII* site, whereas the unaffected members do not, establishing linkage of this mutation to the disease phenotype in this family (Fig. 4c). The mutation creates a frame shift that results in a premature termination codon 8 amino acids beyond the deletion (Fig. 4d). The predicted truncated protein would lack the membrane spanning and cytoplasmic domains of endoglin.

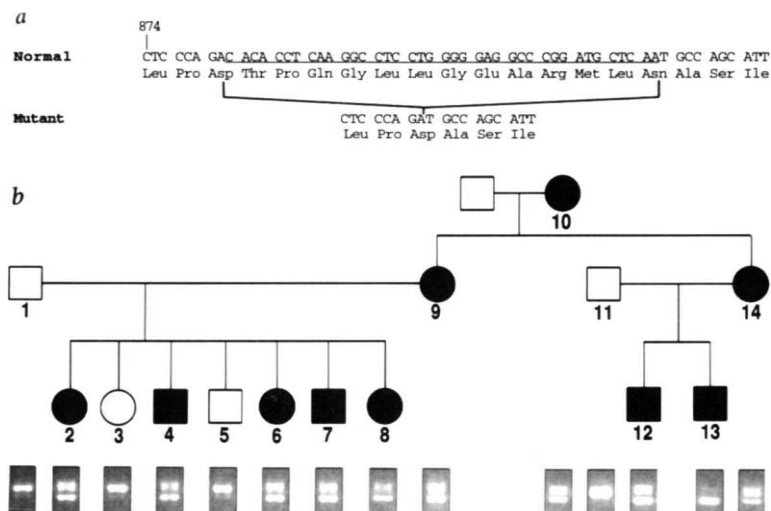


Fig. 3 a, Deletion mutation in sample 8019. a, This 39 bp deletion, found in the 9q3-linked Family 3186 (ref. 9), is located at nucleotide positions 882 through 920 in exon 7, removing 13 amino acids from the protein and altering the first amino acid (position 307) in a potential N-linked glycosylation site. b, Segregation of 39 bp deletion in Family 3186. Amplification of exon 7 in Family 3186 on an agarose gel reveals the presence of a lower band (the 39 bp deletion product) in affected family members only. Preferential amplification of the smaller fragment is sometimes observed (see individual 13).

Discussion

We have established endoglin as the *OWR1* disease locus mapping to 9q3 with the identification of three independent mutations in affected HHT individuals. The gene maps to the tightest *OWR1* candidate interval on 9q33–q34 based on evidence from the mouse and human genetic and physical maps. The restricted tissue distribution of endoglin and its expression at high levels on the surface of endothelial cells is consistent with the pathology of the disorder. Two of the three mutations described in this report create premature termination codons and would be expected to lead to reduced message levels that if translated would encode severely truncated proteins, suggestive of loss-of-function alleles. The third would remove 13 amino acids from the extracellular domain of the receptor and would likely have a deleterious effect on receptor function. Finally, a defect in a cell surface binding protein would account for the limited and localized nature of the vascular lesions present in this disease.

TGF- β *in vivo* is a potent angiogenic factor and a

mediator of vascular remodelling as it controls extracellular matrix production by endothelial cells, smooth muscle cells and pericytes²². Following soft tissue injury or in response to angiogenic factors, microvascular endothelial cells detach from their basement membrane, migrate and proliferate in the interstitial stroma, and form new microvessels. When grown *in vitro* in three-dimensional gels and in the presence of TGF- β , these endothelial cells form tube-like cellular aggregates with a lumen and tight junctions, and deposit an organized basement membrane mimicking vessel formation²². However, TGF- β , almost exclusively in the β 1 isoform, will inhibit the proliferation of endothelial cells grown on plastic^{22,23}. The response of endothelial cells to TGF- β depends on the interaction with the surrounding extracellular matrix via integrins expressed on their surface²⁴. The production of matrix proteins by stromal interstitial cells, smooth muscle cells, pericytes and endothelial cells and the expression of integrins on endothelial cells are also regulated by TGF- β ^{22,24}.

We would expect that endothelial cells lacking endoglin would respond poorly to TGF- β 1 and form abnormal vessels, particularly in response to injury. TGF- β signaling is mediated by TGF- β receptors RI and RII, which form a heteromeric complex upon binding TGF- β ^{25,26}. Endoglin binds TGF- β 1 and - β 3 with high affinity but does not bind - β 2 (ref. 12), and is structurally related to betaglycan which binds all three isoforms of TGF- β ^{27,28}. Betaglycan in the presence of ligand interacts with the signaling kinase complex of RI and RII and potentiates the response to all three isoforms of the growth factor²⁹. Endoglin also interacts with the kinase complex suggesting a potentiating role similar to that of betaglycan. Endothelial cells express very low levels of betaglycan, which may explain their poor response to TGF- β 2 (ref.30). Thus endoglin-deficient endothelial cells, as observed in *OWR1*-linked patients, would only express the signaling RI and RII complex and would lack the regulatory co-receptor capable of controlling the response. This might alter cell adhesion properties, leading to the vascular anomalies seen in this disorder. Stromal cells in several tissues²¹ and activated monocytes¹⁹ also express endoglin and could be impaired in their response to TGF- β 1 in the vascular lesions of *OWR1* patients.

The biological consequences of a defect within any member of the TGF- β ligand-receptor complex are only beginning to be elucidated. TGF- β 1 null mice were initially found to die within three weeks of birth from severe

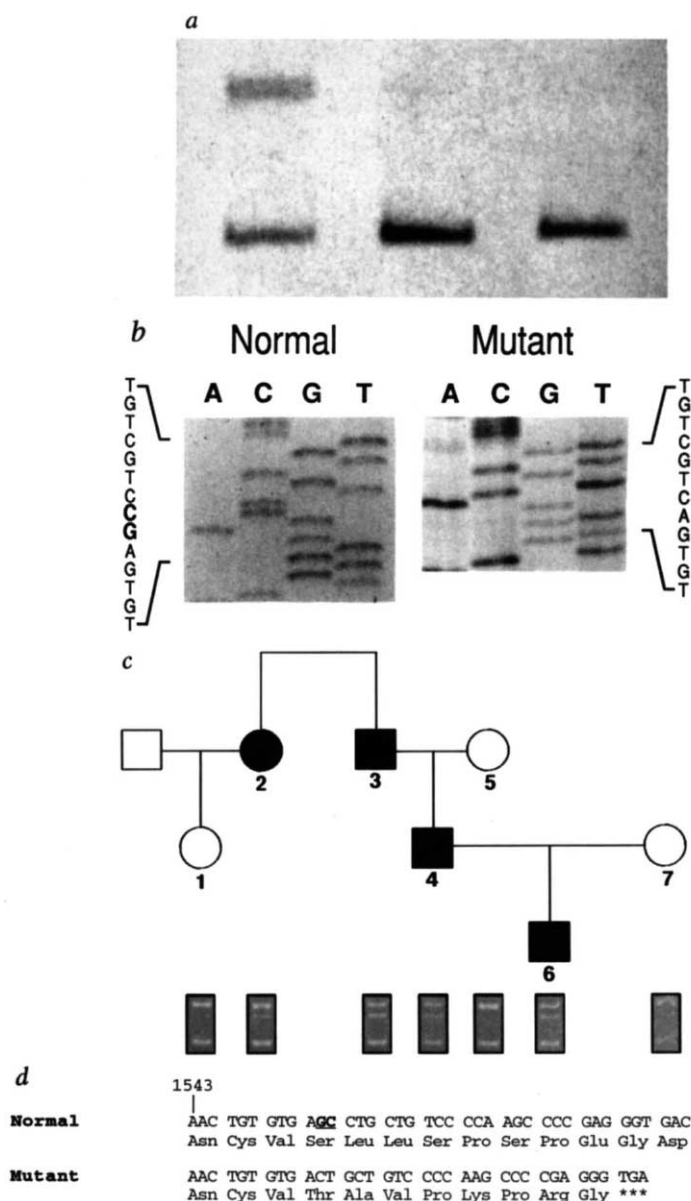


Fig. 4 Deletion mutation in sample 2061. *a*, Heteroduplex analysis showing a shift in an affected proband (sample 2061; lane 1) next to two samples (lanes 2 and 3) not displaying this anomaly. *b*, Sequence of 2 bp deletion in sample 2061. Sequence of the two independently cloned PCR products of affected individual sample 2061 revealing the normal sequence and the 2 bp deletion in exon 11 beginning at nucleotide position 1553. *c*, Segregation of 2 bp deletion in family 63. The 2 bp deletion creates an additional *Mae*III restriction site. Affected family members exhibit an additional novel fragment visible as the middle band in each lane, with half the intensity of the other two bands. A second novel band produced by digestion at this site is not visible on this gel. *d*, Consequence of the 2 bp deletion in sample 2061. This mutation causes a frameshift and a premature termination after an additional seven amino acids.

inflammatory disease^{31,32}. However, maternal TGF- β 1 was later shown to contribute to the survival of the embryos. When a null female was treated with dexamethasone to prevent the inflammatory response and mated to a heterozygous male, the null offspring showed abnormal heart formation with unusual atrioventricular junctions and disordered myocyte proliferation³³.

Our results are particularly significant because they show a direct link between a human genetic disorder and defined mutations within the TGF- β binding protein endoglin. As HHT is a genetically heterogeneous disease, the observation that endoglin is defective in *OWR1*-linked families suggests that loci encoding other components of the TGF- β ligand-receptor complex might explain the locus heterogeneity. Before determining the genomic structure of endoglin for mutation analysis, we performed genetic linkage analysis on three non-9q3-linked families, using genetic markers located near the map positions of the TGF- β ligands (β 1, β 2 and β 3) and the only other mapped TGF- β receptor, the TGF- β type II receptor. One of these families was linked to 3p22 (D.W.S. *et al.*, manuscript in preparation), where the TGF- β II receptor is located³⁴. This supports our hypothesis that the locus heterogeneity in this disorder may be due to mutations within other members of the TGF- β receptor complex or other endothelial cell components of the TGF- β signal transduction pathway.

Continued analysis of endoglin mutations in *OWR1*-linked families will be necessary to determine the functional consequences of the mutations with regard to binding of endoglin with its ligands or disruption of the interactions of endoglin with other TGF- β receptors. There were no obvious differences in the clinical features seen in the three families with described mutations, suggesting that the molecular pathology for the potential loss-of-function mutations (premature termination) and the small deletion may be the same. We favour the hypothesis that *OWR1*, although inherited as an autosomal dominant disorder, exhibits a cellular-recessive pathology and requires inactivation of the normal allele as the initiating event in the formation of a vascular lesion. In support of this, the vascular lesions in this disorder are localized to discrete regions within the affected tissue, with no evidence of abnormal vessel structure or pathology outside the lesions themselves. Cutaneous lesions are most often located in exposed areas that might be subject to ultraviolet irradiation, and in some cases seem to increase in number with age. This hypothesis can be tested by uncovering a secondary (somatic) mutation or loss of heterozygosity in the wild-type allele in endothelial cells of an *OWR1*-derived vascular lesion. Alternatively, the initiating event in the formation of a vascular lesion might be damage to the vessel wall. In this scenario, mutations resulting in reduced endoglin expression might lead to defective repair of the vessel wall. Mutations disrupting endoglin dimerization might lead to a similar outcome due to a dominant-negative effect.

Methodology

Clinical evaluation. The diagnostic criteria used for collection of family members was as described^{5,7}. Descriptions and pedigrees of all 9q3-linked families have been published^{5,7,9}.

Genomic sequence determination. A nearly complete cDNA sequence of endoglin (18A) was used to screen a gridded chromosome 9 cosmid library (Los Alamos National Laboratory). One subclone that contained nearly all hybridizing bands that are seen with genomic DNA was subcloned using Lambda ZAP Express system (Stratagene). Plaque screens were performed by hybridization with 18A cDNA probe to identify positive clones. Intron-exon borders were identified by sequencing these clones using Sequenase Ver. 2.0 DNA sequencing kit (United States Biochemical) using both vector and exon primers.

PCR amplification of exons. Primers were designed from intron genomic sequences flanking exons 7 and 11 of endoglin. For exon 7 (nts 817-992), the forward primer is 5'-GAGGCCTGG-CATAACCCT, and the reverse primer is 5'-GTGGCCA-CTGATCCAAGG. The 315 bp product was amplified using a buffer consisting of 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, and 50 mM KCl. After initial denaturation, 35 cycles of the following program were run: 94 °C for 30 s; 60 °C for 60 s; 72 °C for 30 s. For exon 11 (nts 1429-1686), the forward primer is 5'-ACTCAGGGGTG-GGAACCTCTT and the reverse is 5'-CCTTCCATGCAAACACAG. The 430 bp product was amplified in 10 mM Tris-HCl, pH 8.3, 1 mM MgCl₂, 50 mM KCl and 5 mM NH₄Cl. After initial denaturation, 32 cycles of the following program were run: 94 °C for 30 s; 57 °C for 60 s; 72 °C for 30 s.

Ten of the other 14 exons can be amplified using conditions described in Table 1. Each amplification reaction contains 100 ng of genomic DNA, 100 ng of each oligonucleotide primer, 0.20 mM of each dNTP, 1.25 U of *Taq* DNA polymerase in final volume of 25 μ l. Reaction conditions were optimized individually for each primer pair by adjusting annealing temperatures and buffer conditions as described³⁵, or using the *Taq* polymerase buffer supplied by Boehringer Mannheim Biochemicals, Indianapolis, IN (BMB).

Mutation analysis. Heteroduplex analysis was carried out as described using MDE gel mix (AT Biochem) with the addition of 15% urea. Samples were denatured for 5 min and allowed to slow cool before fragments were separated by electrophoresis on non-denaturing gels. Products were visualized by ethidium bromide staining. Altered PCR products detected by heteroduplex analysis were directly sequenced using AmpliTaq Cycle sequencing kit (Perkin Elmer). Primers were end-labelled and samples run on 6% polyacrylamide gels. PCR products of the individuals containing the identified stop codon and the 2 bp deletion were cloned into pCR-Script Direct SK(+) cloning vector using pCR-Script Direct SK(+) Directional Cloning Kit (Stratagene) and sequenced using Sequenase Ver. 2.0 (United States Biochemical).

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