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ABSTRACT

Background: Hereditary haemorrhagic telangiectasia (HHT) is a genetic disorder present in 1 in 8000 people and associated with arteriovenous malformations. Genetic testing can identify individuals at risk of developing the disease and is a useful diagnostic tool.

Methods: We present an optimised strategy to detect mutations that predispose to HHT. Our strategy includes quantitative multiplex PCR, sequence analysis, RNA analysis, validation of missense mutations by amino acid conservation analysis for the ENG (endoglin) and ACVRL1 (ALK1) genes, and analysis of an ACVRL1 protein structural model. If no causative ENG or ACVRL1 mutation is found, proband samples are referred for sequence analysis of MADH4 (associated with a combined syndrome of juvenile polyposis and HHT).

Results: We summarize data obtained over the last 8 years and describe 16 novel mutations. We identified mutations in 155 of 194 families with confirmed clinical diagnosis (80% sensitivity). Of 155 mutations identified, 94 are in ENG (61%), 58 in ACVRL1 (37%) and 3 in MADH4 (2%).

Discussion: The 39 families (20%) yet to be resolved may carry ENG, ACVRL1, or MADH4 mutations too complex or difficult to detect, or mutations in genes yet to be identified. For most missense variants of ENG and ACVRL1 reported to date, study of amino acid conservation showed good concordance between prediction of altered protein function and disease occurrence.

Keywords: vascular disease, endoglin, ACVRL1, MADH4

INTRODUCTION

Hereditary haemorrhagic telangiectasia (HHT) is an autosomal dominant disorder manifested in 1/8,000 individuals worldwide.¹ Most affected individuals develop epistaxis before age 20.² However, age of onset, incidence and severity are highly variable,³ individuals may not be diagnosed until a life-threatening complication presents. Arteriovenous malformations (AVMs) can occur in pulmonary, cerebral, and hepatic circulation leading to stroke, internal haemorrhage and severe anemia.²⁻⁵

Two genes are causally related to HHT. Mutations in the 30kb *endoglin* (*ENG*; OMIM 187300) gene, associated with a high prevalence of pulmonary AVMs (PAVMs),⁶ lead to HHT1.⁷ Mutations in the 15kb *activin receptor-like kinase-1* gene (*ACVRL1*; ALK-1, OMIM 600376) lead to HHT2,⁸ which is characterized by a lower frequency of pulmonary and cerebral AVMs than HHT1, but which may have a higher incidence of liver involvement.^{6,9} As in other diseases with a high new mutation rate,¹⁰ most families with HHT have a unique mutation, rendering molecular diagnosis labour-intensive. A total of 168 *ENG* and 138 *ACVRL1* mutations of all types have been reported.¹¹⁻¹³ Two additional genes have been associated with HHT recently. Mutations in the *MADH4* tumour suppressor gene have been associated with a combined syndrome of juvenile polyposis and HHT (JPHT; OMIM 175050).¹⁴ An unidentified HHT3 gene linked to chromosome 5 is also likely to account for a subset of HHT patients.¹⁵

We present a strategy for mutation detection in families clinically diagnosed with HHT. We document 80% test sensitivity in mutation identification. We report 16 novel mutations and 7 new polymorphisms. We evaluate the use of evolutionary conservation analysis to predict the effect of missense variants on protein function and disease association.

MATERIALS AND METHODS

Further details on the methods used are found in the online supplemental files.

Patient Samples

Samples for 291 HHT families were referred from Canada and several other countries. Informed consent was obtained from each family according to institutional guidelines for clinical testing and research studies. Research procedures were approved by the Ethics Committee of the Research Institute of the Hospital for Sick Children.

DNA Methods

Total genomic DNA was extracted from peripheral blood lymphocytes (PBL) with the Puregene (Gentra) kit, according to the manufacturer's instructions.

QM-PCR, with some modifications to previously reported conditions,^{16,17} was used to screen for changes in exon size and copy number in all 15 exons of *ENG* and 9 coding exons of *ACVRL1*. The promoter and exons of *ENG*, and exons 2 to 10 of *ACVRL1*, were sequenced. Sequencing was often performed in duplexes, where two exons were sequenced simultaneously. QM-PCR fragments and sequencing chromatograms were analysed using GeneObjects software (Visible Genetics, Inc). Haplotype analysis (by polymorphic markers d12S1677, d12S368, d12S1712, and d12S347) was used to refine analysis for some families.

When no mutation was found, either in *ENG* or *ACVRL1*, samples with appropriate consent were referred to the Marchuk lab at Duke University for *MADH4* sequence analysis. Each *MADH4* mutation identified was confirmed in our clinical laboratory.

RNA and Protein Methods

When required and possible, RT-PCR analysis was performed using a fresh blood sample, gene-specific primers, and inclusion of a puromycin step. Endoglin expression in peripheral blood activated monocytes and umbilical vein endothelial cells was analysed by 35S-methionine labelling and immunoprecipitation using monoclonal antibodies P3D1 and P4A4, as described.¹⁶⁻²⁰

Missense Analysis

Missense variations were analysed to identify disease-causing mutations. First, the literature was searched for reports with sufficient evidence to conclude that the variation is causative. Analysis was completed for all other exons of *ENG* and *ACVRL1* to ensure that no other mutation was present. Evolutionary conservation analysis of *ENG* and *ACVRL1* variants was performed using the SIFT tool.²¹⁻²³ To predict the effect of *ACVRL1* missense variations on protein structure, several were analysed by molecular modelling, as described previously.²⁴

RESULTS

Summary of mutation analysis

Of 291 families referred for analysis, 24 were excluded because the clinical information was insufficient to confirm a diagnosis, and 73 were excluded due to inadequate DNA. The remaining 194 families were analysed by a combination of QM-PCR, duplex sequencing, long PCR and RNA sequencing. We identified mutations in 155 of 194 families (Table 1), resulting in 80% mutation detection sensitivity. Of all mutations identified, 61% are in *ENG* (HHT1), 37% in *ACVRL1* (HHT2), and 2% in *MADH4* (JPHT).

All types of mutations were identified in the *ENG* and *ACVRL1* genes. In our series, missense mutations were associated more frequently with HHT2 (62%) than HHT1 (27%), while splice site variants were associated more frequently with HHT1 (13%) than HHT2 (2%). Nonsense mutations occur with roughly equal frequency (15%). Small deletions and insertions were more common in HHT1 than in HHT2 (Table 1).

Table 1 Mutation Spectrum for 153 HHT families

Mutation Type	<i>ENG</i> (%)	<i>ACVRL1</i> (%)	<i>MADH4</i> (%)	All (%)	Methods ^a
Missense	25 (27)	36 (62)	3 (100)	64 (41)	S
Nonsense	14 (15)	9 (15)	0	23 (15)	S
Splice	12 (13)	1 (2)	0	13 (8)	S
small deletions	22 (23)	4 (7)	0	26 (17)	S, Q
small insertions	12 (13)	3 (5)	0	15 (10)	S, Q
indels	2 (2)	1 (2)	0	3 (2)	
whole-exon and multi-exon deletions and duplications	7 (7)	4 (7)	0	11 (7)	Q
Total	94 (100)	58 (100)	3 (100)	155 (100)	

^a Methods used for mutation detection: S = sequencing, Q = QM-PCR

Seven *ENG* and four *ACVRL1* mutations (7% overall) were whole-exon deletions or duplications identified by QM-PCR and not by sequencing. This study is the first to report whole exon deletions in the *ACVRL1* gene (exons 3 to 8, exon 10, exons 9 and 10). QM-PCR also detected intra-exonic deletions and insertions (36% of *ENG* mutations and 12% of *ACVRL1* mutations), which were also detectable by sequencing. Many of the mutations included here for sensitivity analysis were reported previously^{16-20, 24-26} and are not discussed in the text. Table 2 summarizes novel mutations and polymorphisms found in our cohort.

Table 2 Novel mutations identified

Family	Gene	Mutation Type	Exon	Nucleotide Change	Amino Acid Change	Phenotype ^a
163	<i>ENG</i>	splice	1	c.67+5G→A	p.M1fs	E, T, F
520	<i>ENG</i>	splice	7	c.817-7C→G (IVS6-7C→G)	premature termination	E, T, P
524	<i>ENG</i>	deletion	7	c.896del186	premature termination	E, F
505	<i>ENG</i>	insertion	6	c.715_716insG	p.E239fs	E, T, P, F
508	<i>ENG</i>	insertion	7	c.953_954insC	p.P318fs, p.R333X	P, F
6 & 516	<i>ENG</i>	insertion	7	c.982_983insGCCT	p.S328fs	E, T, G, F
543	<i>ENG</i>	insertion	10	c.1347_1348insCT	p.F450fs, p.L490X	P
202	<i>ENG</i>	multi-exon duplication	2 to 4	c.68_523dup (dup exon 2 to 4)	p.G23_Q174dup	T, P, C
521	<i>ENG</i>	missense	7	c.923C→A	p.A308D	E, T, P, F
534	<i>ACVRL1</i>	splice	4	c.314-3C→G (IVS3-3C→G)	premature termination	E, T, F
214	<i>ACVRL1</i>	nonsense	6	c.716G→A	p.W239X	E, T, F
140 & 510	<i>ACVRL1</i>	multi-exon deletion	3 to 8	g.5365_9652del4288	p.G21_G416del	G, P, F
535	<i>ACVRL1</i>	whole-exon deletion	10	del exon 10	p.V460_Q503del	E, T, G, F
544	<i>ACVRL1</i>	multi-exon deletion	9 & 10	del exons 9 and 10	p.G416_Q503del	E, F
542	<i>ACVRL1</i>	missense	3	c.293A→G	p.N98S	T, H, F
514	<i>ACVRL1</i>	missense	8	c.1055C→A	p.A352D	E, F

^aProband phenotype: E = epistaxis, T = telangiectases, P = pulmonary AVM, C = cerebral AVM, G = gastrointestinal bleeds, H = hepatic AVM, F = family history.

Novel *ENG* mutations

Study of 94 HHT1 families revealed 75 unique mutations; nine previously unreported mutations are described briefly and shown in Table 2 with the clinical phenotype of the proband. Supplementary Figure 1 illustrates the position of these mutations on the mRNA diagrams.

Three novel mutations affecting *ENG* splice sites were identified. In family 163, the +5 G to A substitution changed the Shapiro-Senapathy²⁷ splice score from 76.8 to 62.4, predicting missplicing of exon 1. Family history for the proband included a brother and father with PAVMs. In family 520, the -7 C to G substitution in intron 6 activated a cryptic AG acceptor site, shown by RNA analysis to cause an insertion of CATTAG, leading to a premature stop. In family 524, deletion of 186 nucleotides at position 896 of exon 7 removed the 3' splice site and part of intron 7. This mutation likely causes skipping of exon 7, leading to a frameshift. The proband, whose father had a pulmonary haemorrhage of unknown cause, presented with epistaxis as the only clinical sign.

We identified 4 novel small *ENG* insertions leading to frameshift mutations (Table 2). In family 508, the proband and mother were each diagnosed with PAVMs. In families 6 and 516, later shown to have a common ancestor, there were three generations of affected individuals.

The proband of family 202 had both pulmonary and cerebral AVMs. QM-PCR analysis showed 2.7 copies of exon 4, and 2.9 copies for each of exons 3 and 2, revealing a duplication of *ENG* exons 2 to 4. Figure 1A illustrates one of the QM-PCR reactions. RNA amplification spanning exons 1 through 6 confirmed the presence of a larger transcript (Figure 1B). Sequencing of the transcript confirmed the duplication of exons 2, 3 and 4 leading to a larger protein with in-frame insertion of G23 to Q174. This mutation was also analysed at the protein level (Figure 1C). Immunoprecipitation of endoglin from metabolically-labelled activated PBL revealed that the normal surface glycoprotein (E; 90kDa monomer) was reduced in the proband sample to an estimated $57 \pm 10\%$ of normal levels. A mutant 110kDa monomer (band M) was observed representing a protein with an additional 151 amino acids.

A novel *ENG* missense mutation was found in family 521: the c.923C→A substitution in exon 7 results in a p.A308D conversion. A308 is not highly conserved across species, and this substitution was considered “tolerated” by SIFT. N307 is predicted to be an N-glycosylation site. It is possible that the A308D change could affect the glycosylation of N307 because the negatively charged side chain of aspartic acid could have unfavourable interactions with the negatively charged oligosaccharides. However, the NetNGlyc program (R. Gupta, E. Jung and S. Brunak, in preparation) showed little effect of the A308D substitution (*data not shown*). Nevertheless, two unaffected family members did not carry this substitution, while three affected members did. The probability that the observed pattern occurs by random chance alone is less than 4%. Furthermore, this variant was not observed in 200 normal alleles sequenced for exon 7, supporting A308D as a disease-causing mutation.

Novel *ACVRL1* mutations

Within the *ACVRL1* gene we have identified a total of 43 unique mutations in 58 resolved families. Six of these mutations are novel and are listed in Table 2. Detailed descriptions of some of the mutations are listed below.

In family 534, a splice site mutation (C to G at the -3 position of intron 3), decreased the Shapiro-Senapathy²⁷ splice score from 84 to 72. RT-PCR showed two mutant transcripts: c.314_315ins208 included all of intron 3, and c.314_336del23, caused by a cryptic AG site in exon 4 resulting in deletion of 23 nucleotides (*data not shown*).

QM-PCR identified the first whole-exon deletions to be reported for the *ACVRL1* gene. Families 140 and 510 (no known relationship) had an identical deletion of exons 3 to 8. Figure 2A shows the QM-PCR of all *ACVRL1* coding exons, with a single copy of exons 3 to 8 in the proband. The breakpoints were identified using long PCR with primers spanning the deletion, followed by sequencing, which confirmed an identical direct connection between introns 2 and 8 in the

probands of each family (g.5365_9652del4288, Figure 2B). Haplotype analysis (Figure 2C) suggests that these two families share a common ancestor.

The proband of family 535 had a deletion of *ACVRL1* exon 10 and a confirmed diagnosis of HHT. Five family members showed correlation between presence of the deletion and clinical manifestations. The proband of family 544 experienced frequent nosebleeds and had several affected relatives. A deletion of exons 9 and 10 was found. The deletions in both families were confirmed by QM-PCR using a number of distinct alternate primer sets flanking exons 9 and 10. Additionally, both samples showed a single copy of D12S1677, a short tandem repeat located in intron 9. Both deletions extend 3' of the *ACVRL1* gene and their breakpoints were not identified.

A missense mutation, c.293A→G, was present in the proband of family 542, who had telangiectases and severe liver AVMs necessitating a liver transplant. NetNGlyc analysis predicted that the N98S substitution would completely remove a potential N-glycosylation site at N98 and create a new one at residue N96. None of over 300 normal alleles sequenced for exon 3 showed this variant.

In family 514, an A352D substitution changed a highly conserved non-polar hydrophobic residue to a negatively charged hydrophilic one. Testing this mutant on a model of the ALK1 (*ACVRL1*) protein, based on the 3D structure of ALK5,²⁴ suggests that in this variant, D352 will form new hydrogen bonds with V353 and A327, with possible steric clashes with I326. Since both I326 and A327 are part of the catalytic segment, the substitution is likely to interfere with substrate binding.

Novel Polymorphisms in *ENG* and *ACVRL1* Genes

We found two additional *ENG* polymorphic variants, present in up to 2% of the population and five new *ACVRL1* polymorphic variants (Table 3). The single base pair substitutions in the coding sequence of *ACVRL1* exons 6 and 10 are silent and have a frequency of about 1%. Three intronic polymorphisms, two in intron 5 and one in intron 3, are far from exon boundaries in regions not usually sequenced. The 21-oligonucleotide deletion in intron 5 was found in 5 of 10 HHT families, and in 5 of 10 non-HHT control families.

Table 3 Novel Polymorphisms in *ENG* and *ACVRL1*

Gene	Exon/ Intron	Nucleotide change	Frequency	Amino Acid Change
<i>ENG</i>	exon 10	c.1374A→G	2/100	P458P
<i>ENG</i>	intron 9a	IVS9a-4G→A	1/80	-
<i>ACVRL1</i>	intron 3	IVS3-35A→G	nd	-
<i>ACVRL1</i>	intron 5	g.6969C→T (IVS5-53C→T)	nd	-
<i>ACVRL1</i>	exon 10	c.1452G→A	1/144	R484R
<i>ACVRL1</i>	exon 6	c.666C→T	1/140	H222H
<i>ACVRL1</i>	intron 5	g.6766del21 (IVS5+110del21)	10/20	-

nd = not determined; frequency expressed in terms of individuals tested.

Characterization of Missense Variants

We used an evolutionary conservation tool to analyse all missense variants (both mutations and polymorphisms previously reported and those first described in the current study) in order to validate their potential as disease-causing mutations. A total of 110 (41 *ENG*, 69 *ACVRL1*) variants were investigated using the SIFT tool²². A partial alignment of proteins similar to human endoglin is shown in Supplementary Figure 2 to illustrate the evolutionary conservation and the position of some variants. Table 4 shows a summary of the results. Reliable SIFT predictions (either affecting or tolerated) were available for 101 variants (92%), while predictions for the remaining 9 variants were non-informative. Of 102 mutations reported, 82 (20 *ENG* and 62 *ACVRL1*) were predicted to affect the protein function, and 12 to be tolerated. For example, in *ENG*, SIFT correctly predicts L194P to be a causative mutation because L194 is conserved among all known species of endoglin and betaglycan (TGFBR3) proteins. R197Q was predicted to be benign because Q197 was observed in *ENG* rat and mouse orthologs. Among 8 known polymorphisms with informative SIFT analysis, four were predicted to be benign and three to affect protein function (Table 4).

There was no informative SIFT prediction available for three substitutions of the initiator methionine of endoglin and for T5M and L8P of the leader peptide because there were no corresponding sequences available for SIFT analysis. However, mutations in the initiation codon lead to null alleles, and are causally related to disease. Variants L107R and V125D were predicted to affect the protein function and variants G331S and C382W to be tolerated; however, their MSC scores were greater than 3.25 and considered non-informative SIFT predictions. RT-PCR analysis showed, however, that the mutation associated with G331S (an alteration of the final nucleotide of exon 7) caused exon 7 skipping.

Table 4 SIFT results for ENG and ACVRL1 missense variants

Gene	Type ^a	Number	Variants ^b	SIFT prediction
<i>ENG</i>	M	20	L32R, V49F, G52V, G52D, C53R, W149C, A160D, P165L, L194P, L221P, W261R, I263T, V311G, C363Y, K374S, F403S, S407N, C412S, G413V, S615L	Affecting
<i>ENG</i>	M	6	W196R, G214S, D264N, L306P, A308D , V504M	Tolerated
<i>ENG</i>	M	8	M1V, M1T, M1R, L8P, L107R, V125D, G331S, C382W	Non-informative

<i>ENG</i>	P	2	P131L, D366H (0.5%)	Affecting
<i>ENG</i>	P	4	G191D(6.4%), R197Q(0.9%), P352L, I575T	Tolerated
<i>ENG</i>	P	1	T5M (3.8%)	Non-informative

<i>ACVRL1</i>	M	62	G48R, C51Y, R67W, C77W, N96D, G211D, E215K, G223R, K229R, L273P, I276T, S284F, L285F, S305P, A306P, G309S, H314Y, R329H, D330N, D330Y, S333I, L337P, N341K, C344Y, C344F, A347P, G350R, A352P, A352D , R374W, R374Q, Y375H, M376V, M376R, P378H, P378L, E379K, V380G, R386H, D397N, D397G, I398N, W399S, A400D, G402S, W406C, E407D, R411W, R411Q, R411P, P424T, P424S, P424L, F425V, F425L, P433S, M438T, V441M, P452L, R479L, R484W, K487T	Affecting
<i>ACVRL1</i>	M	6	G48E, W50C, R67Q, N98S , A128D, D179A	Tolerated
<i>ACVRL1</i>	M	0	None	Non-informative

<i>ACVRL1</i>	P	1	A482V	Affecting
<i>ACVRL1</i>	P	0	None	Tolerated
<i>ACVRL1</i>	P	0	None	Non-informative

^a M=Mutation; P=Polymorphism. ^b Novel mutations are in bold. Numbers in parentheses indicate frequency of previously reported polymorphic variants in our study.

DISCUSSION

We describe a strategy that combines QM-PCR, bi-directional sequencing, RT-PCR and missense analysis to identify mutations for 155 of 194 families with a confirmed clinical diagnosis of HHT. We previously showed that genetic testing, if applied in a systematic and optimized program, renders care more effective and less expensive than clinical management alone.²⁸ Test sensitivity for the cost-benefit study was estimated at 75% before our strategy was clinically implemented. Actual test sensitivity of 80% suggests that cost savings from systematic genetic testing for HHT families may be even greater than previously indicated.

QM-PCR is invaluable in detecting whole-exon or multi-exon deletions and duplications that cannot be identified by sequencing and represent 7% of *ENG* mutations and 7% of *ACVRL1* mutations found in our series. To our knowledge, this is the first report of whole-exon or multi-exon deletions in any HHT2 families. QM-PCR analysis is also a more efficient means to identify intra-exonic deletions and insertions than sequencing, because several exons are screened simultaneously. Of the mutations identified, 43% of *ENG* mutations and 19% of *ACVRL1* mutations could be detected by QM-PCR. It is often difficult to distinguish missense mutations from relatively rare polymorphic variants that have not been identified in the general population. We evaluated the use of SIFT analysis to predict variations that affect protein function based on evolutionary conservation. We confirmed that 82 of 102 mutations are predicted to affect protein function. In the case of *ACVRL1*, there is very good correlation

between putative disease-causing mutations and prediction based on evolutionary conservation; SIFT predicted that 62 of 68 missense variants affect the protein function, while the other 6 are tolerated. In the case of *ENG*, 20 of 34 missense variants were predicted to affect protein function, while six were tolerated. There were far fewer proteins in the *ENG* alignment compared to *ACVRL1*. Several non-informative predictions were observed for *ENG* (9), five of these were for variants in the leader peptide, for which very few sequences were available for comparison. For variant G331S, RNA analysis revealed a splice site mutation, which overrides the SIFT prediction. This leaves only 3 non-informative SIFT predictions for *ENG*. SIFT predictions are subject to non-trivial false-positive and false-negative rates and must be used with caution.

Our strategy failed to find mutations for 39 clinically confirmed probands. Some of these patients are likely to have mutations in the putative HHT3 gene, not yet identified but located on chromosome 5.¹⁵ A few families might have undetected *MADH4* mutations, since this gene was only analysed for a subset of clinically confirmed HHT families for whom no *ENG* or *ACVRL1* mutation was found. New evidence suggests that HHT patients should all be tested for *MADH4* mutations if no *ENG* or *ACVRL1* mutation is found because individuals who carry *MADH4* mutations may not present with the classic signs of JPHT (GI involvement or juvenile polyposis) but as HHT patients.²⁹

We also cannot rule out that distant mutations, not readily detected by our strategy and perhaps in unidentified regulatory regions, may affect any of the above genes. To confirm the locus involved in disease, linkage analysis requires several consenting individuals from informative families. Such data is not often available. Sequence analysis of the *ACVRL1* promoter and exon 1 may increase our sensitivity. To date, we have sequenced the *ENG* promoter for 32 samples without finding any mutations.

It is possible that our cohort includes patients that do not have HHT. Clinical diagnosis of HHT is complicated by substantial variation in visceral manifestations, even among individuals with the same mutation, and by age-dependent manifestations of visible signs. This ambiguity encourages specialists to refer patients for genetic testing who have a suspected diagnosis of HHT, some of whom may not carry a genetic predisposition. The number of individuals in the cohort who truly carry a mutation that leads to HHT cannot be determined with certainty; this complicates calculation of test sensitivity. In our series, no mutation was found for 63 families after complete analysis. For 24 families, clinical diagnosis was deemed to be uncertain, either by the referring physician or because the patient had less than three Curaçao criteria.³⁰

A study of HHT patients in the Netherlands reports sensitivity of 90% after sequence analysis alone.³¹ The difference in sensitivity may result from a number of factors other than the molecular diagnostic strategy. In the Dutch study, a relatively homogeneous cohort of patients was studied for as long as 30 years by the same team of physicians, using standardized diagnostic criteria. Our cohort of patients was very heterogeneous in terms of location and physicians, and we could only eliminate those who did not meet the Curaçao criteria. In addition, founder mutations appeared at a greater rate in the Dutch study than in ours.

Despite these complications, molecular testing for HHT families has several real benefits. Once a familial mutation is identified, relatives at risk can be tested conclusively by one efficient and relatively inexpensive test. Individuals with a mutation are identified for intensive clinical surveillance, while those without a mutation may safely be removed from clinical screening.

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COMPETING INTERESTS

There are no competing interests to report.

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Hereditary Haemorrhagic Telangiectasia (HHT) Mutation database:

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Figure Legends

Figure 1 *ENG* mutation identified by QM-PCR and confirmed by RT-PCR.

A duplication of *ENG* exons 2, 3 and 4 was found in family 202. (A) QM-PCR results for control and proband samples: peaks correspond to the exons indicated in italics. Peak height for control samples were set to two copies; in the proband sample, copy numbers were calculated for each exon by GeneObjects™ fragment analysis tool and are shown below the tracing. (B) RT-PCR products amplified using a forward primer outside exon 1 and a reverse primer within exon 6 were run on an agarose gel. The control sample shows a strong band corresponding to the normal transcript (785bp) and a fainter lower band (approximately 650bp) corresponding to an alternately spliced product found in all normal samples tested to date. The proband sample does not contain this smaller band but a larger one (1238 bp), corresponding to the transcript with the duplication. (C) Endoglin protein was immunoprecipitated in duplicate, from metabolically labelled peripheral blood activated monocytes of proband and control. Reduced levels of the normal bands and an additional 110kDa band that corresponds to a protein with additional amino acids coded by the in frame duplication. Key: L: 100bp ladder; C: control sample; P: proband sample; E: normal surface endoglin protein, 90kDa; *p*: intracellular precursor, 80kDa; M: mutant protein, 110kDa.

Figure 2 Analysis of a novel *ACVRL1* multi-exon deletion.

(A) QM-PCR analysis of all *ACVRL1* coding exons in a single reaction, revealed one copy of exons 3 to 8 in the proband of family 510 relative to the control. (B) Long PCR followed by sequencing shows intron 2 joined to intron 8, in samples from the probands of families 510 and 140, indicating an identical deletion. The sequence shows only the deletion product, which is preferentially amplified. (C) Pedigree and haplotypes of family 510 and (D) family 140 show that the allele 189/208 linked to the *ACVRL1* mutation is present in both families. Key: C: control sample; P510: proband family 510; P140: proband family 140.



