ORIGINAL ARTICLE

Hereditary haemorrhagic telangiectasia: mutation detection, test sensitivity and novel mutations

N L Prigoda, S Savas, S A Abdalla, B Piovesan, D Rushlow, K Vandezande, E Zhang, H Ozcelik, B L Gallie, M Letarte

....

See end of article for authors' affiliations
....

Correspondence to:
Dr Michelle Letarte, The Hospital for Sick Children, 555 University Avenue, Toronto, ON, M5G 1X8 Canada; mablab@ sickkids.ca

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.

Objective: To present a strategy for mutation detection in families clinically diagnosed with HHT.

Methods: An optimised strategy for detecting mutations that predispose to HHT is presented. The strategy includes quantitative multiplex polymerase chain reaction, 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: Data obtained over the past eight years were summarised and 16 novel mutations described. Mutations were identified in 155 of 194 families with a confirmed clinical diagnosis (80% sensitivity). Of 155 mutations identified, 94 were in *ENG* (61%), 58 in *ACVRL1* (37%), and three in *MADH4* (2%).

Conclusions: 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. 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.

Hereditary haemorrhagic telangiectasia (HHT) is an autosomal dominant disorder manifested in 1/8000 individuals worldwide.[1] Most affected individuals develop epistaxis before the age of 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 can occur in the pulmonary, cerebral, and hepatic circulation leading to stroke, internal haemorrhage, and severe anaemia.[2–5]

Two genes are causally related to HHT. Mutations in the 30 kb *endoglin* (*ENG*; OMIM 187300) gene, associated with a high prevalence of pulmonary arteriovenous malformations (PAVMs),[6] lead to HHT1.[7] Mutations in the 15 kb *activin receptor-like kinase-1* gene (*ACVRL1*; ALK-1, OMIM 600376) lead to HHT2,[8] which is characterised by a lower frequency of pulmonary and cerebral arteriovenous malformations 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. In all, 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 seven new polymorphisms. We evaluate the use of evolutionary conservation analysis to predict the effect of missense variants on protein function and disease association.

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 with the Puregene (Gentra) kit, according to the manufacturer's instructions.

Quantitative multiplex polymerase chain reaction (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 nine coding exons of *ACVRL1*. The promoter and exons of *ENG*, and exons 2 to 10 of *ACVRL*, were sequenced. Sequencing was often done 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

....

Table 1 Mutation spectrum for 153 HHT families

Mutation type	ENG (%)	ACVRL1 (%)	MADH4 (%)	All (%)	Methods*
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)	S, Q
Whole exon and multiexon deletions and duplications	7 (7)	4 (7)	0	11 (7)	Q
Total	94 (100)	58 (100)	3 (100)	155 (100)	

*Methods used for mutation detection: S = sequencing; Q = quantitative multiplex polymerase chain reaction.

Marchuk laboratory 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, reverse transcriptase polymerase chain reaction (RT-PCR) analysis was undertaken using a fresh blood sample, gene specific primers, and the inclusion of a puromycin step. Endoglin expression in peripheral blood activated monocytes and umbilical vein endothelial cells was analysed by ^{35}S-methionine labelling and immunoprecipitation using monoclonal antibodies P3D1 and P4A4, as described elsewhere.[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 carried out using the SIFT ("sorting intolerant from tolerant") 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 because of 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% were 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 often with HHT2 (62%) than with HHT1 (27%), while splice site variants were associated more often with HHT1 (13%) than with HHT2 (2%). Nonsense mutations occurred with equal frequency (15%). Small deletions and insertions were more common in HHT1 than in HHT2 (table 1).

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 intraexonic 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 have been reported before[16–20 24–26] and are not discussed in the text. Table 2 summarises novel mutations and polymorphisms found in our cohort.

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. (The supplementary figures can be viewed on the journal website (http://www.jmedgenet.com/supplemental/.)

Table 2 Novel mutations identified

Family	Gene	Mutation type	Exon	Nucleotide change	Amino acid change	Phenotype*
163	ENG	Splice	1	c.67+5G→A	p.M1fs	E, T, F
520	ENG	Splice	7	c.817-7C→G (IVS6-7C→G)	Premature termination	E, T, P
524	ENG	Deletion	7	c.896del186	Premature termination	E, F
505	ENG	Insertion	6	c.715_716insG	p.E239fs	E, T, P, F
508	ENG	Insertion	7	c.953_954insC	p.P318fs, p.R333X	P, F
6 and 516	ENG	Insertion	7	c.982_983insGCCT	p.S328fs	E, T, G, F
543	ENG	Insertion	10	c.1347_1348insCT	p.F450fs, p.L490X	P
202	ENG	Multiexon duplication	2 to 4	c.68_523dup (dup exon 2 to 4)	p.G23_Q174dup	T, P, C
521	ENG	Missense	7	c.923C→A	p.A308D	E, T, P, F
534	ACVRL1	Splice	4	c.314-3C→G (IVS3-3C→G)	Premature termination	E, T, F
214	ACVRL1	Nonsense	6	c.716G→A	p.W239X	E, T, F
140 and 510	ACVRL1	Multiexon deletion	3 to 8	g.5365_9652del4288	p.G21_G416del	G, P, F
535	ACVRL1	Whole exon deletion	10	del exon 10	p.V460_Q503del	E, T, G, F
544	ACVRL1	Multiexon deletion	9 and 10	del exons 9 and 10	p.G416_Q503del	E, F
542	ACVRL1	Missense	3	c.293A→G	p.N98S	T, H, F
514	ACVRL1	Missense	8	c.1055C→A	p.A352D	E, F

*Proband phenotype: C = cerebral arteriovenous malformation; E = epistaxis; F = family history; G = gastrointestinal bleeds; H = hepatic arteriovenous malformation; P = pulmonary arteriovenous malformation; T = telangiectases.
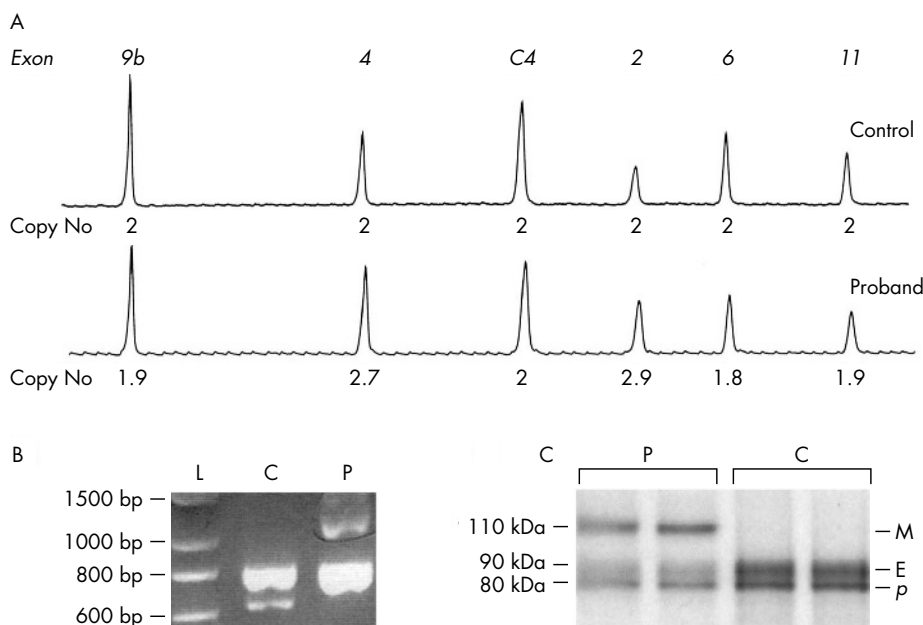
Figure 1 *ENG* mutation identified by quantitative multiplex polymerase chain reaction (QM-PCR) and confirmed by reverse transcriptase polymerase chain reaction (RT-PCR). A duplication of *ENG* exons 2, 3, and 4 was found in family 202. (A) QM-PCR results for control and proband samples: peaks correspond 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 (785 bp) and a fainter lower band (approximately 650 bp) 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 110 kDa band that corresponds to a protein with additional amino acids coded by the in frame duplication. bp, base pair; C, control sample; E, normal surface endoglin protein, 90 kDa; L, 100 bp ladder; M, mutant protein; 110 kDa; P, proband sample; *p*, intracellular precursor, 80 kDa.

Three novel mutations affecting *ENG* splice sites were identified. In family 163, the +5 G to A substitution changed the Shapiro-Senapathy[27] 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 probably 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 four 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 arteriovenous malformations. 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 (fig 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 (fig 1C). Immunoprecipitation of endoglin from metabolically labelled activated peripheral blood lymphocytes showed that the normal surface glycoprotein (E; 90 kDa monomer) was reduced in the proband sample to an estimated 57 (10)%

(mean (SD)) of normal levels. A mutant 110 kDa 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 resulted 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 (Gupta R, Jung E, Brunak S, 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-Senapathy[27] 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).
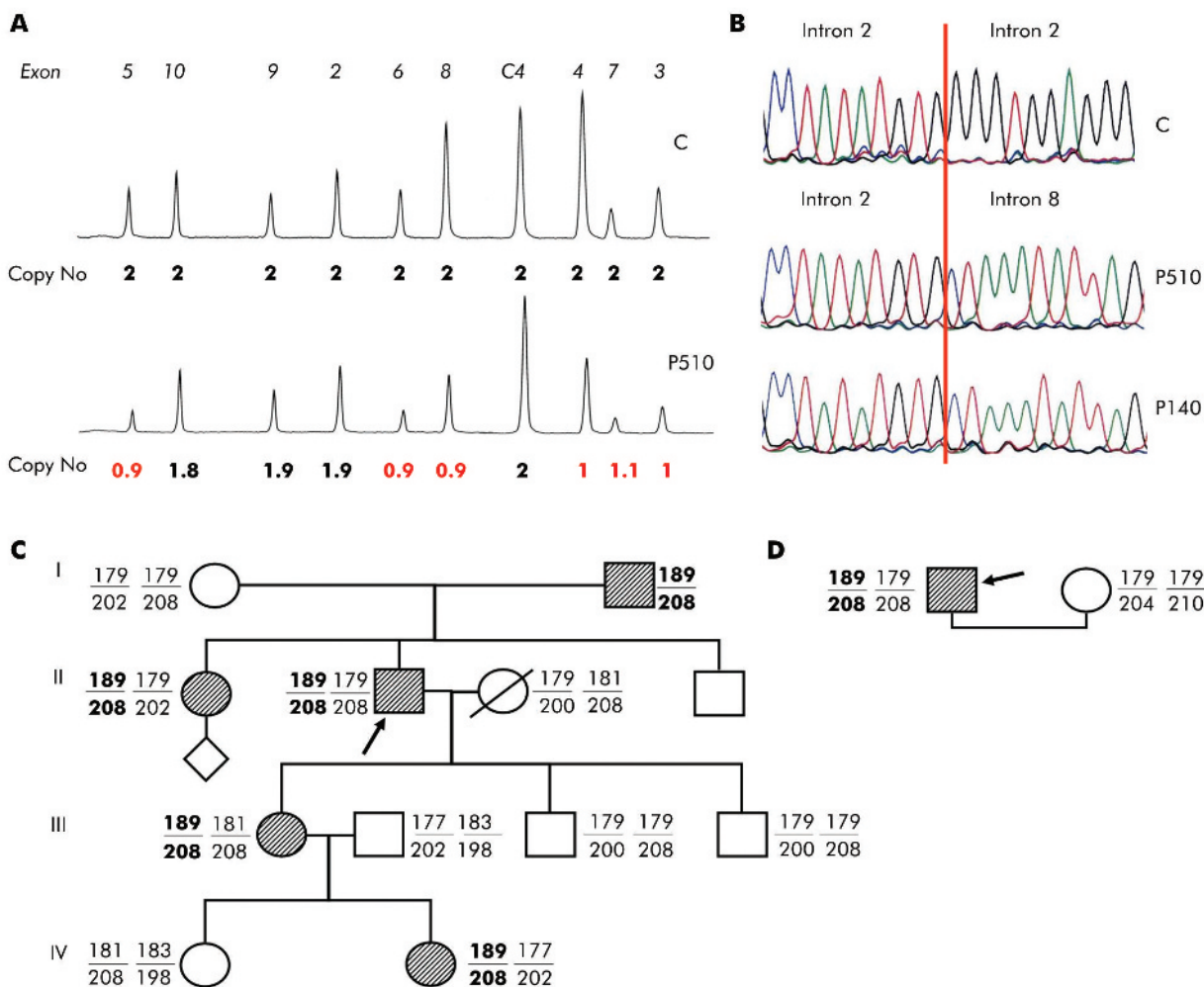
Figure 2 Analysis of a novel *ACVRL1* multiexon deletion. (A) Quantitative multiplex polymerase chain reaction (QM-PCR) analysis of all *ACVRL1* coding exons in a single reaction, revealed one copy of exons 3 to 8 in the proband of family 510 relative to the control. (B) Long PCR followed by sequencing 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. C, control sample; P510, proband family 510; P140, proband family 140.

QM-PCR identified the first whole exon deletions to be reported for the *ACVRL1* gene. Families 140 and 510 (no known relation) 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, fig 2B). Haplotype analysis (fig 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.

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

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

Frequency expressed in terms of individuals tested.
nd, not determined.

Table 4 SIFT results for ENG and ACVRL1 missense variants

Gene	Type*	Number	Variants†	SIFT prediction
ENG	M	20	L32R, V49F, G52V, G52D, C53R, W149C, A160D, P165L, L194P, L221P, W261R, I263T, V311G, C363Y, K374S, F403S, S407N, C412S, G413V, S615L	Affecting
ENG	M	6	W196R, G214S, D264N, L306P, **A308D**, V504M	Tolerated
ENG	M	8	M1V, M1T, M1R, L8P, L107R, V125D, G331S, C382W	Non-informative
ENG	P	2	P131L, D366H (0.5%)	Affecting
ENG	P	4	G191D (6.4%), R197Q (0.9%), P352L, I575T	Tolerated
ENG	P	1	T5M (3.8%)	Non-informative
ACVRL1	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
ACVRL1	M	6	G48E, W50C, R67Q, **N98S**, A128D, D179A	Tolerated
ACVRL1	M	0	None	Non-informative
ACVRL1	P	1	A482V	Affecting
ACVRL1	P	0	None	Tolerated
ACVRL1	P	0	None	Non-informative

*M = mutation; P = polymorphism.
†Novel mutations are in bold. Numbers in parentheses indicated frequency of previously reported polymorphic variants in our study.

A deletion of exons 9 and 10 was found. The deletions in both families were confirmed by QM-PCR using several 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 arteriovenous malformations 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 three dimensional 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. As 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 were silent and had a frequency of about 1%. Three intronic polymorphisms, two in intron 5 and one in intron 3, were far from exon boundaries in regions not usually sequenced. The 21-oligonucleotide deletion in intron 5 was found in five of 10 HHT families, and in five of 10 non-HHT control families.

Characterisation 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. In all, 110 variants (41 ENG, 69 ACVRL1) were investigated using the SIFT tool.[22] A partial alignment of proteins similar to human endoglin is shown in supplementary fig 2 (see the journal website: http://www.jmedgenet.com/supplemental) to illustrate the evolutionary conservation and the position of some variants. Table 4 gives a summary of the

results. Reliable SIFT predictions (either affecting or tolerated) were available for 101 variants (92%), while predictions for the remaining nine 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 orthologues. Among eight 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 to be 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.

DISCUSSION
We describe a strategy that combines QM-PCR, bidirectional 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 optimised programme, 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. An 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 multiexon 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 multiexon deletions in any HHT2 families. QM-PCR analysis is also a more efficient means of identifying intraexonic 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 a 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 six 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 than in *ACVRL1*. Nine non-informative predictions were observed for *ENG*; 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 three 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, as 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 (gastrointestinal tract involvement or juvenile polyposis) but as HHT patients.[29]

We also cannot rule out the possibility 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 are not often available. Sequence analysis of the *ACVRL1* promoter and exon 1 may increase the detection sensitivity. To date, we have sequenced the *ENG* promoter for 32 samples without finding any mutations.

It is possible that our cohort includes patients who do not have HHT. Clinical diagnosis of HHT is complicated by large 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 have 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 the 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 fewer than three Curaçao criteria.[30]

A study of HHT patients in the Netherlands reported sensitivity of 90% after sequence analysis alone.[31] The difference in sensitivity may result from several 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 standardised diagnostic criteria. Our cohort of patients was very heterogeneous in terms of location and physicians involved, 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.

ELECTRONIC DATABASE INFORMATION

Electronic URL addresses for the databases and algorithms used in this article are as follows:

GenBank: http://www.ncbi.nih.gov/GenBank/
NetNGlyc: http://www.cbs.dtu.dk/services/NetNGlyc/
SIFT: http://blocks.fhcrc.org/sift/SIFT_seq_submit2.html
Swiss-Prot/TrEMBL: http://au.expasy.org/sprot/
HHT Mutation database: http://137.195.14.43/cgi-bin/WebObjects/hht.woa/1/wo/yeJpFl44ZUVy9ScN5xDn90/16.3.11

The supplementary tables are present on the journal website (http://www.jmedgenet.com/supplemental).

· · · · · · · · · · · · · · · · · · ·

Authors' affiliations

N L Prigoda, B Piovesan, D Rushlow, K Vandezande, E Zhang, B L Gallie, HHT Solutions, Toronto Western Hospital, Toronto, Canada
S Savas, H Ozcelik, Fred A Litwin Centre for Cancer Genetics, Samuel Lunenfeld Research Institute and Department of Pathology and Laboratory Medicine, Mount Sinai Hospital; Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada
S A Abdalla, Department of Laboratory Medicine and Pathobiology, St Michael's Hospital, Toronto, Canada
M Letarte, Cancer Research Program, The Hospital for Sick Children, Heart and Stroke Richard Lewar Center of Excellence and Departments of Immunology and Medical Biophysics, University of Toronto, Toronto, Canada

Conflicts of interest: none declared

REFERENCES

1 **Guttmacher AE**, Marchuk DA, White RI. Hereditary hemorrhagic telangiectasia. *N Engl J Med* 1995;**333**:918–24.
2 **Plauchu H**, de Chadarevian JP, Bideau A, Robert JM. Age-related clinical profile of hereditary hemorrhagic telangiectasia in an epidemiologically recruited population. *Am J Med Genet* 1989;**32**:291–7.
3 **Shovlin CL**, Letarte M. Hereditary haemorrhagic telangiectasia and pulmonary arteriovenous malformations: issues in clinical management and review of pathogenic mechanisms. *Thorax* 1999;**54**:714–29.
4 **Maher CO**, Piepgras DG, Brown RD, Friedman JA, Pollock BE. Cerebrovascular manifestations in 321 cases of hereditary hemorrhagic telangiectasia. *Stroke* 2001;**32**:877–82.
5 **Buscarini E**, Danesino C, Plauchu H, de Fazio C, Olivieri C, Brambilla G, Menozzi F, Reduzzi L, Blotta P, Gazzaniga P, Pagella F, Grosso M, Pongiglione G, Cappiello J, Zambelli A. High prevalence of hepatic focal nodular hyperplasia in subjects with hereditary hemorrhagic telangiectasia. *Ultrasound Med Biol* 2004;**30**:1089–97.
6 **Letteboer TG**, Mager HJ, Snijder RJ, Koeleman BP, Lindhout D, Ploos van Amstel HK, Westermann KJ. Genotype–phenotype relationship in hereditary hemorrhagic telangiectasia. *J Med Genet* 2005;**43**:371–7.
7 **McAllister KA**, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J, *et al.* Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. *Nat Genet* 1994;**8**:345–51.

8 **Berg JN**, Gallione CJ, Stenzel TT, Johnson DW, Allen WP, Schwartz CE, Jackson CE, Porteous ME, Marchuk DA. The activin receptor-like kinase 1 gene: genomic structure and mutations in hereditary hemorrhagic telangiectasia type 2. *Am J Hum Genet* 1997;**61**:60–7.

9 **Kuehl HK**, Caselitz M, Hasenkamp S, Wagner S, el-Harith HA, Manns MP, Stuhrmann M. Hepatic manifestation is associated with ALK1 in hereditary hemorrhagic telangiectasia: identification of five novel ALK1 and one novel ENG mutations. *Hum Mutat* 2005;**25**:320.

10 **Richter S**, Vandezande K, Chen N, Zhang H, Sutherland J, Anderson J, Han L, Panton R, Branco P, Gallie B. Sensitive and efficient detection of RB1 gene mutations enhances care for families with retinoblastoma. *Am J Hum Genet* 2003;**72**:253–69.

11 **Abdalla SA**, Letarte M. Hereditary haemorrhagic telangiectasia: current views on genetics and mechanisms of disease. *J Med Genet* 2006;**43**:97–110.

12 **Argyriou L**, Pfitzmann R, Wehner LE, Twelkemeyer S, Neuhaus P, Nayernia K, Engel W. ALK-1 mutations in liver transplanted patients with hereditary hemorrhagic telangiectasia. *Liver Transpl* 2005;**11**:1132–5.

13 **Lenato GM**, Lastella P, Di Giacomo MC, Resta N, Suppressa P, Pasculli G, Sabba C, Guanti G. DHPLC-based mutation analysis of ENG and ALK-1 genes in HHT Italian population. *Hum Mutat* 2006;**27**:213–4.

14 **Gallione CJ**, Repetto GM, Legius E, Rustgi AK, Schelley SL, Tejpar S, Mitchell G, Drouin E, Westermann CJ, Marchuk DA. A combined syndrome of juvenile polyposis and hereditary haemorrhagic telangiectasia associated with mutations in MADH4 (SMAD4). *Lancet* 2004;**363**:852–9.

15 **Cole SG**, Begbie ME, Wallace GM, Shovlin CL. A new locus for hereditary haemorrhagic telangiectasia (HHT3) maps to chromosome 5. *J Med Genet* 2005;**42**:577–82.

16 **Cymerman U**, Vera S, Pece-Barbara N, Bourdeau A, White RI, Dunn J, Letarte M. Identification of hereditary hemorrhagic telangiectasia type 1 in newborns by protein expression and mutation analysis of endoglin. *Pediatr Res* 2000;**47**:24–35.

17 **Cymerman U**, Vera S, Karabegovic A, Abdalla S, Letarte M. Characterization of 17 novel endoglin mutations associated with hereditary hemorrhagic telangiectasia. *Hum Mutat* 2003;**21**:482–92.

18 **Pece N**, Vera S, Cymerman U, White RI, Wrana JL, Letarte M. Mutant endoglin in hereditary hemorrhagic telangiectasia type 1 is transiently expressed intracellularly and is not a dominant negative. *J Clin Invest* 1997;**100**:2568–79.

19 **Pece-Barbara N**, Cymerman U, Vera S, Marchuk DA, Letarte M. Expression analysis of four endoglin missense mutations suggests that haploinsufficiency is the predominant mechanism for hereditary hemorrhagic telangiectasia type 1. *Hum Mol Genet* 1999;**8**:2171–81.

20 **Paquet ME**, Pece-Barbara N, Vera S, Cymerman U, Karabegovic A, Shovlin C, Letarte M. Analysis of several endoglin mutants reveals no endogenous mature or secreted protein capable of interfering with normal endoglin function. *Hum Mol Genet* 2001;**10**:1347–57.

21 **Ng PC**, Henikoff S. Predicting deleterious amino acid substitutions. *Genome Res* 2001;**11**:863–74.

22 **Ng PC**, Henikoff S. Accounting for human polymorphisms predicted to affect protein function. *Genome Res* 2002;**12**:436–46.

23 **Ng PC**, Henikoff S. SIFT: predicting amino acid changes that affect protein function. *Nucleic Acids Res* 2003;**31**:3812–14.

24 **Abdalla SA**, Cymerman U, Johnson RM, Deber CM, Letarte M. Disease-associated mutations in conserved residues of ALK-1 kinase domain. *Eur J Hum Genet* 2003;**11**:279–87.

25 **Abdalla SA**, Pece-Barbara N, Vera S, Tapia E, Paez E, Bernabeu C, Letarte M. Analysis of ALK-1 and endoglin in newborns from families with hereditary hemorrhagic telangiectasia type 2. *Hum Mol Genet* 2000;**9**:1227–37.

26 **Abdalla SA**, Cymerman U, Rushlow D, Chen N, Stoeber GP, Lemire EG, Letarte M. Novel mutations and polymorphisms in genes causing hereditary hemorrhagic telangiectasia. *Hum Mutat* 2005;**25**:320–1.

27 **Shapiro MB**, Senapathy P. RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. *Nucleic Acids Res* 1987;**15**:7155–74.

28 **Cohen JH**, Faughnan ME, Letarte M, Vandezande K, Kennedy SJ, Krahn MD. Cost comparison of genetic and clinical screening in families with hereditary hemorrhagic telangiectasia. *Am J Med Genet A* 2005;**137**:153–60.

29 **Gallione CJ**, Richards JA, Letteboer TG, Rushlow D, Prigoda NL, Leedom TP, Ganguly A, Castells A, Ploos van Amstel JK, Westermann CJ, Pyeritz RE, Marchuk DA. SMAD4 mutations found in unselected HHT patients. *J Med Genet*, 2006; Apr 13 [Epub ahead of print]..

30 **Shovlin CL**, Guttmacher AE, Buscarini E, Faughnan ME, Hyland RH, Westermann CJ, Kjeldsen AD, Plauchu H. Diagnostic criteria for hereditary hemorrhagic telangiectasia (Rendu-Osler-Weber syndrome). *Am J Med Genet* 2000;**91**:66–7.

31 **Letteboer TG**, Zewald RA, Kamping EJ, de Haas G, Mager JJ, Snijder RJ, Lindhout D, Hennekam FA, Westermann CJ, Ploos van Amstel JK. Hereditary hemorrhagic telangiectasia: ENG and ALK-1 mutations in Dutch patients. *Hum Genet* 2005;**116**:8–16.

Call for abstracts

International Forum on Quality & Safety in Health Care
18–20 April 2007, Palau De Congressos, Barcelona
Deadline: 25 September 2006
http://www.quality.bmjpg.com