Clinical and Analytical Sensitivities in Hereditary Hemorrhagic Telangiectasia Testing and a Report of *de Novo* Mutations

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Hereditary hemorrhagic telangiectasia is a vascular dysplasia with variable onset and expression. Through identification of a mutation in a proband, mutation testing can be offered to family members. Mutation carriers can receive medical surveillance and treatment before potentially fatal complications arise. In this study, we assessed the significance of clinical evaluations as part of hereditary hemorrhagic telangiectasia diagnostic testing to determine the clinical sensitivity of molecular testing and to report novel mutations. Based on reported clinical symptoms, we classified 142 consecutive cases as affected, suspected, or unlikely affected. We performed temperature gradient capillary electrophoresis and full gene sequencing of both *ACVRL1* and *ENG* genes. We then compared the mutation detection rates between these groups, categorizing sequence variants as mutations, variants of uncertain significance (VUS), or known polymorphisms. Our mutation and VUS detection rate in affected individuals was 74% and 16% in the suspected/unlikely affected group. Sixty-one percent of the mutations and all VUS were novel. The mutation detection rate for temperature gradient capillary electrophoresis was 97%. Our results suggest that a careful clinical evaluation increases the mutation detection rate. We have confirmed the occurrence of *de novo* mutations in three patients. Our results also show that temperature gradient capillary electrophoresis is an efficient mutation screening method. (J Mol Diagn 2007, 9:258–265; DOI: 10.2353/jmoldx.2007.060117)

Hereditary hemorrhagic telangiectasia (HHT) is characterized phenotypically by telangiectases and arteriovenous malformations. These lesions result in hemorrhage, particularly in the nose, gastrointestinal tract, and brain, and complications related to shunting, primarily in

the lungs and liver. Complications from this disorder include intracranial hemorrhage secondary to cerebral arteriovenous malformations and embolic stroke and brain abscess secondary to pulmonary arteriovenous malformations. The frequency of HHT is reported to be ~1 in 10,000, but it is thought to be underdiagnosed.¹

Two genes, endoglin (*ENG*) and activin receptor-like kinase 1 (*ACVRL1*), have been reported to cause HHT in an autosomal dominant manner if mutated.² Molecular diagnosis allows for diagnostic confirmation in symptomatic individuals and significantly improves care for individuals at risk for HHT after identification of a causative mutation. Because the initial clinical presentation of the disorder can be a catastrophic pulmonary or central nervous system event,^{3–5} presymptomatic diagnosis for relatives of individuals with HHT offers an opportunity to prevent serious or lethal complications. Individuals shown to be unaffected can be spared unnecessary and costly medical screening. Developing simple and reliable diagnostic approaches has been difficult because of the lack of common mutations.² Thus, sensitive mutation scanning approaches followed by targeted sequencing might be useful in the clinical setting.

To detect mutations many scanning techniques have been developed, differing in sensitivity, specificity, throughput, and cost. In this study, we used temperature gradient capillary electrophoresis (TGCE), which has been described to be reliable in detecting heteroduplexes caused by sequence variants such as point mutations or small deletions and insertions⁶ followed by DNA sequencing.

The mutation detection rate by sequencing of these two genes has been previously reported as 68 to 78%.^{7–9} Deletions/duplications or a third¹⁰/fourth¹¹ locus may account for the rest of the cases. In addition, mutations in *SMAD4* cause manifestations of HHT combined with juvenile polyposis.¹²

In this study, we review 143 consecutive cases received for molecular analysis of HHT genes from January 2004 to April 2006. We present clinical background and

Supplemental material for this article can be found on <http://jmd.amjpathol.org/>.

Accepted for publication November 13, 2006.

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molecular test results obtained by gene scanning and sequencing of *ACVRL1* and *ENG*. We report novel sequence variations and address statistically the proportion of variants of uncertain significance (VUS) detected in affected individuals. In addition, we report three *de novo* mutations for this disease.

Materials and Methods

Cases

Samples from 143 consecutive cases were submitted to Associated Regional and University Pathologists laboratories for clinical HHT testing and included in the study on institutional review board approval. Individuals were grouped into three categories based on the symptoms as described by the ordering physician. It is of note that the policy of our laboratory is to contact an ordering clinician for additional information when HHT mutation analysis is received for an individual who does not meet clinically published diagnostic criteria¹ based on the information provided on our requisition form.

Group 1 consisted of 97 clinically affected individuals who were reported to have at least three of the following criteria: positive family history of HHT, recurring nosebleeds, dermal/oral telangiectases, liver shunts, pulmonary arteriovenous malformations, cerebral arteriovenous malformations, and gastrointestinal telangiectases/arteriovenous malformations.¹ Group 2 consisted of 29 individuals with a suspected diagnosis of HHT, who had two of the above-mentioned criteria. Group 3 consisted of 16 individuals that were considered unlikely to have HHT because they had only one of the above-mentioned criteria. One individual had insufficient clinical information and therefore was not included further in our study groups. This individual had no mutation or VUS.

Mutation Analysis

DNA was obtained from 1 ml of blood by MagnaPure Compact (Roche, Indianapolis, IN) extraction following the manufacturer's instructions. Sample analysis in the clinical laboratory involved polymerase chain reaction (PCR) and heteroduplex formation of all exons in *ACVRL1* (GenBank accession no. AH005451) and *ENG* (GenBank accession no. AH006911) followed by TGCE scanning and targeted sequencing for 20 samples. If no mutation or VUS was found, the remaining exons were sequenced as well. In 123 cases, we did TGCE screening analysis and full gene sequencing simultaneously and compared the results. For potential splice-site variants, mRNA was isolated from whole blood. cDNA was prepared using random primers and amplified using locus-specific primers. Gel electrophoresis was performed to confirm the presence of two mRNA species and sequencing to confirm the alternate splicing.

PCR and TGCE

PCR was performed in 25- μ l reactions using 0.4- μ mol primers (forward and reverse), 1 $\times$ High Fidelity PCR Master (Roche), and 100 ng of DNA. Primer sequences were as described by Bayrak-Toydemir and colleagues.² PCR and heteroduplex formation were performed in a PE 9700 (Applied Biosystems). PCR cycling conditions were 95°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds; 72°C for 7 minutes; and 4°C for 1 minute followed by heteroduplex formation according to manufacturer's recommendation (SpectruMedix Inc., State College, PA).

After heteroduplex formation, 5 μ l of sample was diluted with 1 $\times$ PCR buffer (Applied Biosystems, Foster City, CA) to ensure unsaturated fluorescent intensity and suitable salt concentrations. Diluted samples were injected into a SpectruMedix instrument equipped with 24 capillaries (model SLE 2410; SpectruMedix Inc.) for TGCE. Other parameters included 3 kV (20 seconds) for sample injection, 50 to 60°C for temperature gradient, and 9 kV (50 minutes) for data collection. The ramp period was 21 minutes (from 50 to 60°C).⁶ Data were analyzed using the Revelation 2.4 image analysis software (SpectruMedix Inc.). Peak patterns were used to score individual exons of a sample as wild type if there was only one sharp peak present. They were scored as heteroduplex if there were either more than one peak present or a peak with a shoulder. A peak pattern of four individual peaks indicated an insertion or deletion.

DNA Sequencing

PCR products were purified using ExoSAP-IT (USB, Cleveland, OH). Sequencing was performed using Big Dye Terminator chemistry and either the 3100 Genetic Analyzer or 3730 DNA Analyzer (Applied Biosystems). Sequencing Analysis v.5.0 (Applied Biosystems) and Sequencher (GeneCodes, Ann Arbor, MI) software were used for analysis and mutation detection.

Throughout this article the term mutation is used to refer to sequence variations with convincing evidence to suggest that they are causative of HHT. Such evidence includes causation of a frameshift or premature stop codon, mRNA analysis to confirm splice-site variants, tracking of the sequence variation with the disorder in multiple, preferably distantly related family members, or a publication presenting information that is suggestive of the variation being causative of HHT.^{2,13} We refer to VUS as sequence variants for which this type of evidence does not currently exist to determine whether they are benign or deleterious. Thus, when interpreting the significance of missense mutations, possible splice-site mutations, as well as in-frame small deletions and insertions, we advocate a more conservative approach for clinical laboratories than often used by research laboratories.

SIFT Analysis

Uncertain variants that are point mutations were analyzed using SIFT (Sorting Intolerant from Tolerant), available

Table 1. Mutation Detection Rates in Clinically Affected, Suspected, and Unlikely Affected Individuals

	Affected individuals, n (%)	Suspected individuals, n (%)	Unlikely affected individuals, n (%)	Total, n (%)
Mutation	49 (50.5)	2 (6.9)	0	51 (35.9)
VUS	23 (23.7)	2 (6.9)	3 (18.7)	28 (19.7)
Negative	25 (25.8)	25 (86.2)	13 (81.3)	63 (44.4)
Total	97	29	16	142

from <http://blocks.fhcrc.org/sift/SIFT.html>.¹⁴ SWISS-PROT 48.7 and TREMBL 31.7 were the databases searched with the median conservation of sequences set at 3.00 and sequences more than 90% identical removed. Amino acid changes with scores greater than 0.05 were considered tolerated.

Results

In 142 individuals tested for mutations in the *ACVRL1* and *ENG* genes, 51 (36%) were determined to have mutations, 28 (20%) were determined to have VUS, and no mutation could be detected in 63 individuals (44%). The mutation frequency in all three groups is summarized in Table 1. In clinically affected individuals, we found a 51% (49 of 97) mutation detection rate and a 24% (23 of 97) VUS detection rate. As discussed below, most VUS are likely to be causative, and therefore combining both mutations and VUS gave an overall detection rate of 74% in clinically affected individuals. When suspected and unlikely affected individuals were combined, the mutation detection rate was 4% (2 of 45), and the VUS detection rate was 11% (5 of 45). No mutation or VUS was detected in 84% (38 of 45) of these cases.

Of the 51 individuals with mutations, 25 (49%) had mutations in *ACVRL1* and 26 (51%) in *ENG*. Of the 31 VUS, 13 (42%) occurred in *ACVRL1* and 18 (58%) occurred in *ENG*. Two individuals had both a mutation and a VUS, and one individual had two VUS. Of the 14 mutations in the *ACVRL1*, 11 were missense, two were deletions, and one was a splice-site mutation (Table 2).^{15–25} Among the 13 VUS in *ACVRL1*, 10 were missense variants, two were possible splice-site mutations, and one was an in-frame deletion (Table 3).

Of the 23 mutations in the *ENG*, four were missense, one was nonsense, seven were insertions, five were deletions, five were splice-site mutations, and one was an indel mutation (Table 2). Among the 18 VUS, 15 were missense mutations, and three were in-frame deletions (Table 3). The novel polymorphisms found in this study and their frequencies are shown in Table 4. Three individuals among the 97 positive cases (3.1%) had *de novo* mutations. Both parents were clinically asymptomatic and tested negative for this mutation. Parentage was confirmed for all three cases using short tandem repeat (STR) markers. Two of these cases are described in detail in the discussion.

SIFT analysis of uncertain variants that are point mutations predicted that 87% (20 of 23) are protein altering. Two variants in *ACVRL1*, p.Y88C and p.S167C, and one variant in *ENG*, p.R205P, were predicted to be tolerated amino acid changes with *P* values of 0.20, 0.18, and 0.13, respectively. However, all three of these changes occurred in clinically affected individuals. Two potential splice-site variants in *ENG*, c.219G>A and c.360 + 4_7delAGTG, were further analyzed. Splicing is predicted to be affected in both variants because two species of cDNA were detected and confirmed by sequencing.

Clinical Sensitivity

Most of the detected mutations and VUS occurred in clinically affected individuals (72 of 79, 91%). The detection rate of mutations and VUS combined in clinically affected individuals was 74% (72 of 97) and 16% (7 of 45) in suspected/unlikely affected individuals. Because most VUS (82%, 23 of 28) were found in clinically affected individuals, we suspect that the majority of them are causative mutations. To further analyze this hypothesis, we calculated the likelihood of being affected when a mutation was detected and compared it to the likelihood of being affected when a VUS was detected. To do this, we calculated the posterior probability of an individual being affected by HHT if a mutation is observed from the Table 1 using Bayes' rule.

According to Bayes' rule,

$$P(A = 1|M) = \frac{P(M|A = 1) \times P(A = 1)}{\sum_{i \in 0,1} P(M|A = i) \times P(A = i)}$$

where $A = 1, 0$ represents an individual being affected and not affected, respectively, and $P(M)$ represents the probability of observing a mutation.

From Table 1, the priors and likelihood can be calculated as follows: $P(M|A = 1) = 49/97$ and $P(M|A = 0) = 2/45$. Also $P(A = 1) = 97/142$ and $P(A = 0) = 45/142$. Using the estimates for likelihood, we calculated the probability of being affected as 0.961. This suggests that if a mutation is observed in at least 1 of the 23 exons, it is very likely that the individual is affected by HHT.

Using a similar approach, we arrived at the probability of an individual being affected when a VUS is observed in at least 1 of the 23 exons to be 0.82. This shows that the likelihoods of being affected are similarly high (0.961 versus 0.82), whether a mutation or VUS is found, and it supports our hypothesis that most VUS are likely to be causative.

Analytical Sensitivity of TGCE

TGCE detected 97% of the mutations and VUS. It failed to detect one mutation, c.525 + 1G>A in *ACVRL1*, and one VUS, c.1586G>A in *ENG*. Both of these were detected by full gene sequencing. Several polymorphisms were repeatedly missed by TGCE, most of which were located in introns and located toward the ends of the amplicons. A

Table 2. List of the Mutations Found in This Study

Gene	Mutation type	Nucleotide change	Amino acid change	Number of cases and clinical status	Reference
<i>ACVRL1</i>	Deletion	c.31_50del20 [‡]	p.L11fsX36	1 Affected*	Novel
<i>ACVRL1</i>	Splice site	c.525 + 1G>A	Unknown	1 Affected	Novel
<i>ACVRL1</i>	Deletion	c.759_761delCGA	p.D254del	2 Affected	15
<i>ACVRL1</i>	Missense	c.925G>A	p.G309S	1 Affected	16
<i>ACVRL1</i>	Missense	c.1030T>C	p.C344R	1 Affected	2
<i>ACVRL1</i>	Missense	c.1031G>A	p.C344Y	2 Affected	17
<i>ACVRL1</i>	Missense	c.1048G>C	p.G350R	1 Affected	16
<i>ACVRL1</i>	Missense	c.1120C>T	p.R374W	7 Affected and 1 suspected	18
<i>ACVRL1</i>	Missense	c.1121G>A	p.R374Q	1 Affected	19
<i>ACVRL1</i>	Missense	c.1126A>G	p.M376V	1 Affected	8
<i>ACVRL1</i>	Missense	c.1232G>A	p.R411Q	2 Affected	20
<i>ACVRL1</i>	Missense	c.1232G>C	p.R411P	1 Affected	8
<i>ACVRL1</i>	Missense	c.1435C>T	p.R479X	2 Affected	8
<i>ACVRL1</i>	Missense	c.1436G>A	p.R479Q	1 Affected	2
<i>ENG</i>	Missense	c.1A>G	p.M1V	1 Affected	16
<i>ENG</i>	Insertion	c.34_35insGGCTGTTGCCC	p.L12fsX18	1 Affected	2
<i>ENG</i>	Insertion	c.41_42insTGGCCAGCTGC [‡]	p.S18fsX46	1 Affected	Novel
<i>ENG</i>	Splice site	c.219G>A	p.T73T, skips exon 2, S23fs	1 Affected	Novel [§]
<i>ENG</i>	Missense	c.277C>T	p.R93X	2 Affected and 1 suspected	21
<i>ENG</i>	Splice site	c.360 + 4_7 delAGTG	Unknown	1 Affected	Novel [§]
<i>ENG</i>	Deletion	c.471_472delCT	p.S158fsX160	1 Affected	Novel
<i>ENG</i>	Splice site	c.524-2A>G	p.N121fs	2 Affected	22
<i>ENG</i>	Missense	c.662T>C	p.L221P	1 Affected	23
<i>ENG</i>	Deletion	c.771delC	p.P257fsX358	1 Affected	Novel
<i>ENG</i>	Insertion	c.967insT	p.V323fsX333	1 Affected	34
<i>ENG</i>	Missense	c.991G>A	p.G331S	1 Affected [†]	16
<i>ENG</i>	Splice site	c.992-1G->A	Unknown	1 Affected	Novel
<i>ENG</i>	Deletion	c.995delG	p.G332fsX358	1 Affected	16
<i>ENG</i>	Missense	c.1121_1122AA>GC [‡]	p.K374S	1 Affected	16
<i>ENG</i>	Splice site	c.1135 + 1G>C	p.G332fs	1 Affected	25
<i>ENG</i>	Deletion	c.1267delA	p.N423fsX490	1 Affected	22
<i>ENG</i>	Insertion	c.1311 + 1_2insG	Unknown	1 Affected	Novel
<i>ENG</i>	Indel	c.1415_1417delAGAinsGT	p.Q472fsX490	1 Affected	21
<i>ENG</i>	Insertion	c.1470_1471insA	p.L490fsX500	1 Affected	21
<i>ENG</i>	Insertion	c.1470insGGGTG	p.D491fsX520	1 Affected	Novel
<i>ENG</i>	Insertion	c.1476insC	p.C493fs	1 Affected	Novel
<i>ENG</i>	Deletion	c.1479delC	p.H494fsX517	1 Affected	Novel

*This patient also had c.77 C>T (p.P26L) VUS in the *ACVRL1*.

[†]This patient also had c.1445C>T (p.A482V) VUS in the *ENG*.

[‡]*De novo* mutations.

[§]mRNA confirmation done.

detailed list of these can be found in Supplemental Table 1 at <http://jmd.amjpathol.org/>.

TGCE was especially helpful in one instance, when it indicated a sequence variant; however, the variant was missed during initial sequencing. In retrospect, the variant showed a low-fluorescent signal in the forward direction, below the detection threshold set for the instrument. The reverse reaction had a high enough background to also mask the variant. Because of the TGCE result, the sample was resequenced, and the variant was detected.

Discussion

In this study, we grouped our cases into three categories, namely, clinically affected, suspected affected, and unlikely affected, based on their clinical symptoms to correlate the likelihood of detecting a mutation with the reported clinical criteria for HHT. Our suspicion that individuals with at least three clinical manifestations of HHT, and therefore a clinically confirmed diagnosis, have a higher mutation detection rate (74%) than suspected or

unlikely affected individuals (15%) was confirmed. Mutation detection rates of 68 to 78% have been published for clinically affected individuals⁷⁻⁹; however, recent studies reported that the mutation detection rate can be increased by ~10% when deletion/duplication analysis is added to sequencing.^{9,26,27} We are continuing to follow up known affected individuals with no mutation identified for deletions/duplications that would be missed by sequencing.

Molecular diagnosis of HHT requires a stepwise, family-based approach. An individual who meets clinical diagnostic criteria for HHT should be tested first in each family. Given the high penetrance of this disorder by middle age^{28,29} and the low *de novo* mutation frequency,²⁷ a targeted physical examination, medical history, and family history are more sensitive for making the initial diagnosis of HHT in a family than molecular genetic testing. In light of this, and because sequence analysis is costly, we recommend performing sequencing-based genetic testing for HHT only on clinically well-evaluated patients.

Table 3. List of the Variants Uncertain Significance Found in This Study

Gene	Location	Nucleotide change	Amino acid change	Number of cases and clinical status	SIFT score
ACVRL1	Exon 3	c.263 A>G	p.Y88C	1 Affected	Tolerated, 0.20
ACVRL1	Exon 3	c.269 G>A	p.C90Y	1 Affected	Protein affected, 0.00
ACVRL1	Exon 4	c.500C>G	p.S167C	1 Affected	Tolerated, 0.18
ACVRL1	Intron 4	c.525 + 3A>G	Unknown	1 Affected	N/A
ACVRL1	Intron 6	c.773-3C>G	Unknown	1 Affected	N/A
ACVRL1	Exon 7	c.812C>A	p.T271K	1 Affected	Protein affected, 0.00
ACVRL1	Exon 7	c.914C>T	p.S305F	1 Affected	Protein affected, 0.00
ACVRL1	Exon 7	c.982C>T	p.H328Y	1 Affected	Protein affected, 0.00
ACVRL1	Exon 8	c.1112G>T	p.G371V	1 Unlikely affected	Protein affected, 0.00
ACVRL1	Exon 8	c.1220A>G	p.E407G	1 Affected	Protein affected, 0.00
ACVRL1	Exon 10	c.1460_1462delAGA	p.487delK	1 Affected	N/A
ENG	Exon 2	c.95 T>A	p.L32H	1 Affected	Protein affected, 0.00
ENG	Exon 3	c.290 T>G	p.L97R	1 Affected	Protein affected, 0.01
ENG	Intron 3	c.360 + 5G>C	Unknown	1 Affected	N/A
ENG	Exon 4	c.446G>C	p.W149S	1 Suspected	Protein affected, 0.00
ENG	Exon 4	c.494C>G	p.P165R	1 Affected	Protein affected, 0.01
ENG	Exon 5	c.580_591del TCGAGTGGCGGC	p.194_197delLEWR	1 Affected	N/A
ENG	Exon 5	c.614G>C	p.R205P	1 Affected	Tolerated, 0.13
ENG	Exon 5	c.659T>A	p.I220N	1 Unlikely affected	Protein affected, 0.00
ENG	Exon 5	c.659T>C	p.I220T	1 Affected	Protein affected, 0.01
ENG	Exon 5	c.659_660TC>AT	p.I220N	1 Affected*	Protein affected, 0.00
ENG	Exon 7	c.872_883del12bp	p.K291_D294del	1 Affected	N/A
ENG	Exon 7	c.899T>C	p.L300P	1 Affected	Protein affected, 0.01
ENG	Exon 8	c.1087T>A	p.C363S	1 Affected	Protein affected, 0.00
ENG	Exon 8	c.1235G>A	c.C412Y	1 Unlikely affected	Protein affected, 0.00
ENG	Exon 9a	c.1309C>T	p.R437W	1 Affected	Protein affected, 0.00
ENG	Exon 11	c.1586G>A	p.R529H	1 Suspected	Protein affected, 0.01
ENG	Exon 11	c.1595T>C	p.F532S	1 Affected	Protein affected, 0.00
ENG	Exon 13	c.1844C>T	p.S615L	1 Affected*	Protein affected, 0.00

*Same patient has two VUS listed in this table. N/A, not available.

A large number of VUS found in this study are likely causative mutations, given that 82% (23 of 28) of them occurred in clinically affected individuals with no other mutation or variant identified. This is in contrast to other disorders, such as inherited breast cancer with a common phenocopy, in which VUS are much less likely to be causative. Although our statistical analysis showed that a VUS found in a clinically affected person is likely to be a causative mutation, a clinical laboratory must have a high degree of certainty before reporting a missense mutation

or suspected splice-site mutation as a causative mutation rather than a VUS. Methods for assessing whether a VUS is clinically significant include amino acid change analysis, paralog and ortholog comparisons for amino acid conservation, family concordance studies, mRNA analysis, and protein modeling. Comparisons of amino acid change and conservation are simple to do using available computer programs, such as SIFT (<http://blocks.fhcrc.org/sift/SIFT.html>), but by themselves have led to mistakes in predicting the effect of a variant. Our SIFT analysis predicts that 20 of 23 of the uncertain variants that are point mutations affect protein function. Three of these 20 variants occurred in unlikely affected individuals and two of them in suspected affected individuals. All three variants that were predicted to be tolerated amino acid changes occurred in individuals that had a clinically confirmed diagnosis. These results are in favor of our hypothesis that most of our VUS are causative; however, caution should be taken when trying to predict an individual variant's effect on the protein because SIFT has a published false-negative rate of 31% and a false-positive rate of 21%.¹⁴ Unfortunately, methods with greater predictive potential, such as family concordance, mRNA studies, and protein modeling, are time consuming and expensive for clinical laboratories. One question facing clinical laboratories is where the role of a clinical laboratory ends and that of a research laboratory starts. Information produced in research laboratories is frequently used to establish new clinical tests and to interpret the results of these tests. Yet single amino acid substitutions

Table 4. List of Novel Benign Variants or Polymorphisms Found in This Study

Gene	Location	Frequency
ACVRL1	c.314-49G>A	1.4%
ACVRL1	c.330G>A, p.S110S	0.7%
ACVRL1	c.626-59delG	1.4%
ACVRL1	c.773-59insT	2.8%
ACVRL1	c.1048 + 55_58delTCTC	0.7%
ACVRL1	c.1377 + 45T>C*	29.4%
ACVRL1	c.1377 + 65A>G*	29.4%
ACVRL1	c.1512 + 58G>A	2.1%
ENG	c.1-9G>A	0.7%
ENG	c.219 + 22_23CG>GA	0.7%
ENG	c.219 + 25G>A	1.4%
ENG	c.219 + 58insC	2.1%
ENG	c.817-27delCT	0.7%
ENG	c.1135 + 54C>T	0.7%
ENG	c.1407G>A, p.P469P	0.7%
ENG	c.1742-31T>C	0.7%
ENG	c.1932C>T, p.I644I	1.4%

*These two polymorphisms are found in linkage disequilibrium.

in an HHT gene are typically reported in publications by research groups as a mutation if they are not found in 100 chromosomes of unaffected individuals, the change is conserved between species, or has been reported once previously in a patient with HHT. Although most of the novel missense mutations and suspected splice-site mutations detected in HHT patients will be disease causing, we believe the threshold should be higher before they are reported or published as deleterious. This dilemma needs to be considered carefully, particularly when the data are transferred from research studies. Three cases included in this study demonstrate the difficulty of interpreting VUS in a clinical lab setting.

Case 1

One young individual with confirmed HHT but an otherwise negative family history was found to have both a deletion (c.31_50del20) in *ACVRL1*, resulting in a premature stop codon, and a VUS (c.77C>T, p.P26L) in *ACVRL1*, resulting in a single amino acid substitution that had not been previously reported. Parental samples were requested, and the deletion was not detected in either parent. It was thus reported to be an apparent *de novo* mutation in the affected child. The c.77C>T variant was present in the individual's father and was assumed to be a benign variant based on the deletion being found in the affected child and the father's reported lack of HHT symptoms. During follow-up communication with the family's clinician, some questions arose as to whether the father and a paternal uncle did have symptoms suggestive of HHT. The clinician was encouraged to get additional information to clarify the clinical status of the father and uncle, stating that the interpretation of the variant and recurrence risk for the parents would be affected. In the end, a past episode of gastrointestinal bleeding in the father was determined to be unrelated, as was an episode of blood in the spinal fluid of the uncle. Neither had a history of nosebleeds or telangiectasia.

Case 2

An affected individual was found to have two sequence variations in *ENG* (c.1844C>T and c.659_660TC>AT). Kuehl and colleagues³⁰ had previously described c.1844C>T in an individual who also had c.1135G>A (p.E379K) in *ACVRL1*. Thus, the significance of c.1844C>T was considered to be uncertain because it had only been reported in the presence of another sequence variation in an affected individual. It was unclear whether c.659_660TC>AT is a change of two nucleotides on the same chromosome, causing p.I220N, or whether it is 659T>A (p.I220N) on one chromosome and 660C>T (p.I220I) on the other, so it too was classified as a variant of uncertain significance. A family concordance study to evaluate the chromosome phase and whether either variant tracks with HHT in the family has been offered but not pursued by the family to date.

Case 3

An affected individual was found to have c.1121-1122AA>GC in *ENG*. To determine whether both of these nucleotide changes are in *cis*-(p.K374S) or *trans* (p.K374N and p.K374R), DNA testing of this variant was offered to the individual's parents. The mutation was found in neither of the apparently unaffected parents, and STR testing was done to confirm parentage. This mutation therefore is assumed to be *de novo* and causative of HHT.

A clinical laboratory must also balance the desire for a testing protocol that has the highest possible sensitivity with concerns about the cost of testing. Because 23 exons constitute the two currently identified HHT genes, we searched for the best methodology to screen for mutations before sequencing. TGCE is a relatively new method that allows for screening of heteroduplexes that are formed after PCR if a heterozygous mutation is present in a PCR amplicon.⁶ It can detect insertions, deletions, and most point mutations in an amplicon. However, it is unable to detect large deletions or duplications. It is especially useful in scanning genes of autosomal dominant disorders, when only one mutation is expected. In our study, we detected 36 of the 37 unique mutations and 28 of the 29 VUS by TGCE for a sensitivity of 97% (64 of 66). It has also detected a variant that was missed by the initial sequencing. Several polymorphisms, mainly positioned at the ends of amplicons, were missed by TGCE. Factors affecting detection by TGCE include the length of the amplicon, the position of the mutation on the amplicon (end versus middle), and the GC content of the fragment. With increasing fragment length the difference in migration during TGCE of a heteroduplex versus a homoduplex decreases, therefore making it harder to detect heteroduplexes. Because a heterozygous sequence variation is nearer the end of the fragment, its migration during TGCE resembles more that of a homoduplex than a variation in the middle of the fragment that begins to melt the fragment from the middle out. As the GC content of a fragment increases, melting is more inhibited during TGCE; therefore, it can also contribute to the heteroduplex migrating similarly to the homoduplex, making it hard to detect. Overall, TGCE followed by targeted sequencing is an effective strategy for mutation detection in the HHT genes. It saves cost by reducing the number of sequencing reactions from 46 (full gene analysis) to an average of 10 in cases in which a mutation was identified by targeted sequencing. Figure 1 shows examples of TGCE results for wild-type amplicons, point mutations, and an insertion. The mutation that was initially missed by TGCE is shown in Figure 1d. An enlargement was necessary to reveal a slight shoulder on the right of the peak. Detection of these slight shoulders is difficult and requires experienced analysts.

The spontaneous mutations in HHT are rare, with a single report by Cymerman and colleagues.²⁷ We report *de novo* mutations in 3.1% of our samples. This is low compared with many other autosomal dominant diseases

Figure 1. Examples of TGCE results. The x-axis shows the migration time in data points (one data point = 1 second), and the y-axis shows the fluorescent intensity. **a:** Typical wild-type peak, in this case, ACVRL1 exon 10. **b:** Typical peak pattern for a point mutation with two clearly distinguishable peaks, in this case, c.1029C>T in ENG. **c:** Another typical peak pattern for a point mutation. The peak has a shoulder on the right side. This amplicon contained c.207G>A in ENG. **d:** Hard-to-distinguish peak with shoulder. This amplicon had c.525 + 1G>A in ACVRL1. This variant was initially missed by TGCE analysis. After full gene analysis, TGCE data were reanalyzed, and this small shoulder on the right side was evident. This peak was enlarged to show ~85 data points, whereas all of the others show ~160 data points. **e:** Characteristic peak pattern for insertions and deletions. This amplicon contained c.991 + 26_27insCCTCCC in ENG.

but significant enough to pursue clinical testing in individuals with symptoms consistent with HHT but lacking a family history.

In conclusion, we have reported novel mutations and VUS found in our clinical testing, including three *de novo* mutations. A statistical analysis supports the hypothesis that VUS found in affected individuals are likely to be causative. In addition, we report the mutation detection rate for TGCE to be 97%. Because of the complexity of HHT analysis, we recommend that testing be offered to those with a clinical diagnosis of HHT.

Acknowledgment

We thank the clinical DNA Sequencing Laboratory at Associated Regional and University Pathologists for their help.

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