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Mutation analysis of "Endoglin" and "Activin receptor-like kinase" genes in German patients with hereditary hemorrhagic telangiectasia and the value of rapid genotyping using an allele-specific PCR-technique

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Abstract

Background: Hereditary hemorrhagic telangiectasia (HHT), also known as Rendu-Osler-Weber syndrome, is an autosomal dominant disorder which is clinically characterised by recurrent epistaxis, mucocutaneous telangiectasia and visceral arteriovenous malformations. Genetic linkage studies identified two genes primarily related to HHT: endoglin (*ENG*) on chromosome 9q33-34 and activin receptor-like kinase I (*ACVRL1*) on chromosome 12q13. We have screened a total of 41 unselected German patients with the suspected diagnosis of HHT. Mutation analysis for the *ENG* and *ACVRL1* genes in all patients was performed by PCR amplification. Sequences were then compared to the HHT database <http://www.hhtmutil.org> sequences of the *ENG* mRNA (accession no. BC014271.2) and the *ACVRL1* mRNA (accession no. NM000020.1).

Results: We identified 15 different mutations in 18 cases by direct sequencing. Among these mutations, one novel *ENG* mutation could be detected which has not yet been described in the literature before. The genotype-phenotype correlation was consistent with a higher frequency of pulmonary arteriovenous malformations in patients with *ENG* mutations than in patients with *ACVRL1* mutations in our collective.

Conclusion: For rapid genotyping of mutations and SNPs (single nucleotide polymorphisms) in *ENG* and *ACVRL1*, allele-specific PCR methods with sequence-specific primers (PCR-SSP) were established and their value analysed.

Background

Hereditary hemorrhagic telangiectasia (HHT, MIM#18730), also known as Rendu-Osler-Weber syndrome, is an autosomal dominant disorder of the fibrovascular tissue. Worldwide, HHT has a prevalence of 1 in 10.000 individuals [1,2]. This multi-systemic angiogenic disorder is clinically characterized by severe and recurrent hemorrhages due to epistaxis, mucocutaneous telangiectasia and arteriovenous malformations. Vascular lesions are present in two forms, either as telangiectases on the skin as well as the inner lining of the mouth, nose and gastrointestinal tract (GI-tract) and/or as direct arteriovenous shunts most commonly seen in the lung (PAVM), brain (CAVM) and liver (HAVM) [3-9]. Electron microscopy studies on lesion biopsies revealed that dilated postcapillary venules connect directly to arterioles without intervening capillary bed [10,11]. As a result, these vascular lesions can be the cause for severe complications involving gastrointestinal bleedings, the risk of embolism, stroke and abscesses [12].

Till now, HHT can not be cured. The treatment of HHT patients remains symptomatic or has preventive character. The Curaçao criteria are in clinical use for diagnosis of HHT [13]. However, the clinical manifestations of HHT can be very heterogeneous, both between families and among members of the same family, making the diagnosis of the disease at times very difficult.

In the 90s, genetic linkage studies revealed the two major types of disease, HHT1 and HHT2, caused by mutations in the *ENG* (endoglin) and *ACVRL1* genes. The first locus (HHT type 1) was mapped to chromosome 9q33-34 [14,15], where the 40 kb endoglin (*ENG*, OMIM 187300) was defined as the affected gene [16], also associated with a high prevalence of pulmonary arteriovenous malformations [17]. The second locus (HHT type 2) mapping to chromosome 12q was identified as the 15 kb activin receptor-like kinase-1 gene (*ACVRL1*, OMIM 600376) [18,19]. It is characterised by a lower incidence of pulmonary and cerebral arteriovenous malformations than HHT1, but has a higher incidence of liver manifestation [17,20]. The corresponding endoglin and ALK-1 proteins are specific endothelial receptors of the transforming growth factor beta superfamily essential for maintaining vascular integrity [21,22]. Many mutations have been identified in *ENG* and *ACVRL1* genes and support the haploinsufficiency model for HHT [23]. In recent studies, two more genes have been implicated in HHT, which have not yet been identified, the HHT3 gene on chromosome 5 [24], and the HHT4 gene on chromosome 7 [25]. In addition, SMAD4 mutations mapping to chromosome 18 have been observed in patients with a combined syndrome of juvenile polyposis and HHT (JPHT) [26].

In this study, we report mutational analysis of the two main genes *ENG* and *ACVRL1* in a German population affected by HHT. We describe a novel mutation in the *ENG* gene in HHT patients which has not been described before. Furthermore, we determine the value of allele-specific PCR for rapid genotyping.

Methods

Patients and controls

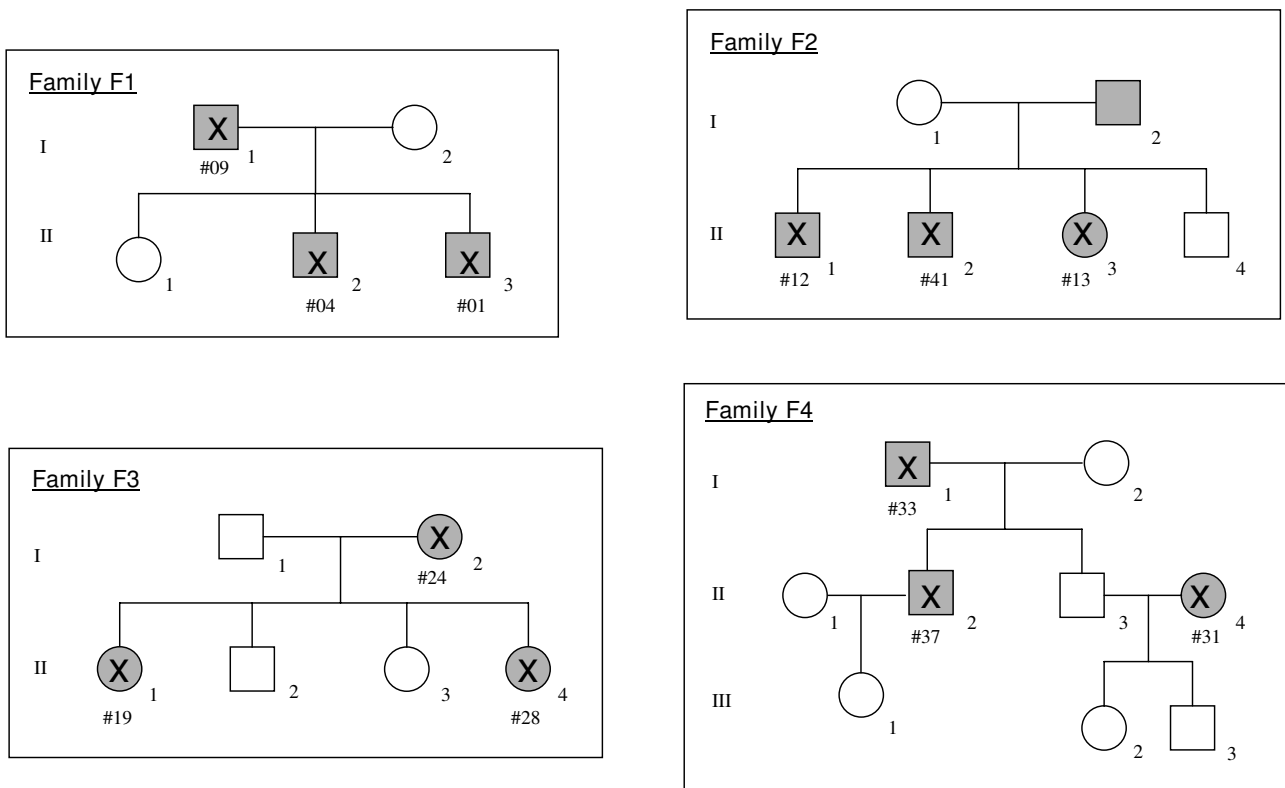
Anti-coagulated peripheral blood samples were obtained from 41 patients of German origin with tentative diagnosis of HHT at the Department of ORL, Head and Neck Surgery, University Hospital Mannheim, and from 768 healthy blood donors as a control group from the Institute of Transfusion Medicine and Immunology, Mannheim (Additional file 1). The HHT patients comprised 4 small-sized families (3 persons each) and 29 single cases (Fig. 1). Information on the clinical data including the medical history, physical examination and diagnostic screening was obtained in personal interviews with the affected individuals and from their patient records with all the data being documented in an HHT database. According to the Curaçao criteria, diagnosis for HHT included the presence of three out of four key symptoms: recurrent epistaxis, telangiectasia, hereditary with an affected first degree relative and visceral involvement [13] (Additional file 2). An informed consent was obtained from each individual and from parents of patients younger than 18 years according to the approval of the Ethic Committee of the University of Mannheim.

Isolation of DNA

DNA was extracted from anti-coagulated peripheral blood samples using a commercial DNA isolation kit (QIAamp DNA blood mini kit; Qiagen, Hilden, Germany) according to the standard protocol.

Sequencing of *ENG* and *ACVRL1* exons

All coding exons and flanking intronic sequences of the *ENG* and *ACVRL1* genes were PCR amplified using primers published previously [27,28]. PCR was performed in a total volume of 50 µl including 100 ng DNA, 0.5 µM each primer, 200 µM each dNTP, standard PCR buffer (Qiagen) and 1 U HotStar Taq DNA polymerase (Qiagen). The cycling parameters were as follows: initial denaturation for 15 min at 95°C; 35 cycles with 20 sec. at 94°C, 30 sec. at 60°C and 60 sec. at 72°C; final extension for 5 min at 72°C. After the PCR, products were run on 2% agarose gels. The 15 exons of *ENG* and the 9 exons of *ACVRL1* were sequenced in 41 patients with HHT using Thermosequenase reagents (GE Healthcare Amersham), IRD700 and IRD800 labelled primers and an automated system (NEN® Global IR² DNA Sequencer; LI-COR inc., Lincoln, NE, USA). Sequences were compared to the HHT database

**Figure 1**

Pedigree of the four small-sized HHT families. White square and white circle = unaffected, grey square and grey circle = affected, X = DNA available, # = Pat.-no.

<http://www.hhtmution.org> sequences of the *ENG* mRNA (accession no. BC014271.2) and the *ACVRL1* mRNA (accession no. NM000020.1).

PCR-SSP typing of SNPs

For rapid genotyping of the mutations and SNPs in *ENG* and *ACVRL1*, allele-specific PCR methods with sequence-specific primers (PCR-SSP) were established. Specific primer sequences and sizes of PCR products are given in Additional file 3. Part of the β -globin locus was amplified as an internal control PCR fragment (540 bp). According to a published PCR-SSP protocol [29,30], 20 ng DNA were subjected to 10 μ l PCR reactions including 0.5 μ M each allele-specific primers, 0.1 μ M each internal control primer, standard PCR buffer (Qiagen), 200 μ M each dNTP and 0.5 units Taq DNA polymerase (Qiagen). The cycling conditions were: 2 min initial denaturation at 94°C, followed by 10 cycles with 20 s denaturation at 94°C and 1 min annealing/extension at 65°C, followed by 20 cycles with 20 s duration at 94°C, 1 min annealing at 61°C and 30 s extension at 72°C. Amplification products were separated on 2% agarose gels containing 0.5 ng/ml ethidium bromide in a rapid agarose gel electrophoresis (RAGE; Cascade Biologics, Inc., Portland, OR, USA) chamber for

5 min at 25 V/cm. Results were obtained by visual inspection of the gels and were documented by using a UV documentation device with CCD camera (UVP, Inc., Upland, CA, USA).

Results

The 15 coding exons of the *ENG* gene and the 9 exons of the *ACVRL1* gene including flanking intron sequences were PCR amplified and sequenced from 41 patients with HHT. The sequence data were compared to each other and to the *ENG* and *ACVRL1* sequences published in databases (HHT Mutation Database: <http://www.hhtmution.org>). Mutations could be detected in 18 cases (43.9%).

Sequence analysis of *ENG*

ENG mutations

We identified 5 different *ENG* mutations in 6 out of 41 HHT patients. The 5 different *ENG* mutations represented: 1 deletion, 1 insertion, 2 splice site mutations and 1 missense mutation (Additional file 4). In our study, mutation c.816+2T>C was detected in 2 related cases. One novel *ENG* mutation was detected in exon 10 (c.1384insT) causing a frameshift (p.Gln462fs) which has not yet been

listed in the HHT gene database before. Altogether, all of the mutations found in *ENG* were detected in exons coding for the extracellular protein-domain. No mutations were detected in the region coding for the intracellular domain (Fig. 2).

Polymorphisms in the *ENG* gene

The 5 different polymorphisms and their relative frequencies identified in the *ENG* gene are presented in Additional file 5. A previously reported G to A substitution at position 207 of exon 2 (c.207G>A) was observed in 12 out of 41 HHT patients (29.3%). Two patients carried a c.1844C>T polymorphism in exon 13 (4.8%). At position 1029 (c.1029C>T) of exon 8, a C to T change was observed in 7 HHT patients (17%). A c.1347A>G polymorphism in exon 10 and a c.1771G>A polymorphism in exon 13 was observed in one HHT patient (2.4%).

Sequence analysis of *ACVRL1*

***ACVRL1* mutations**

Ten different *ACVRL1* mutations could be identified in 12 out of 41 HHT patients, including 2 deletions, 2 insertions, 1 splice site mutation and 5 missense mutations (Additional file 6). The insertion c.144_145insG (p.Ala49fs) was detected in 2 members of the same family, whereas the missense mutation c.1120C>T (p.Arg374Trp) was detected in 2 unrelated cases. Half of the HHT2 associated *ACVRL1* mutations consisted of single base substitutions, leading to amino acid changes. More than 75% of all *ACVRL1* mutations were detected in exons coding for the intracellular domain (Fig. 3). Espe-

cially four out of five missense mutations (80%) were found in the receptor domain or the kinase domain, mainly affecting exon 8. No mutations were detected in the region coding for the transmembrane domain.

PCR-SSP typing for *ENG* and *ACVRL1* mutations

Further investigation on allele and genotype frequencies focused on the mutations identified in our cohort of HHT patients. PCR-SSP systems could be established for all *ACVRL1* mutations and all *ENG* mutations. A panel of DNA samples from HHT patients with different mutations was selected on the basis of the sequencing data and was used to validate all 15 PCR-SSP systems. The genotypes were estimated on the basis of the presence or the absence of the allele-specific PCR products (Fig. 4). All of the 41 HHT samples were retyped by PCR-SSP and data were compared to sequencing data. We could achieve 100% concordance of data, i.e. DNA samples with mutation revealed a positive PCR signal in the corresponding PCR-SSP system and DNA samples without mutation were negative in all 15 PCR-SSP systems. In addition, the 15 PCR-SSP systems were applied for genotyping of the *ENG* and *ACVRL1* mutations in 768 healthy blood donor controls. None of the healthy control individuals showed an *ENG* or *ACVRL1* mutation indicating the specificity of the gene variants for the HHT phenotype.

Phenotype-Genotype Correlation

Diagnosis of HHT in an individual required that they met three or more of the following four Curaçao criteria: 1) spontaneous and recurrent epistaxis, 2) mucocutaneous

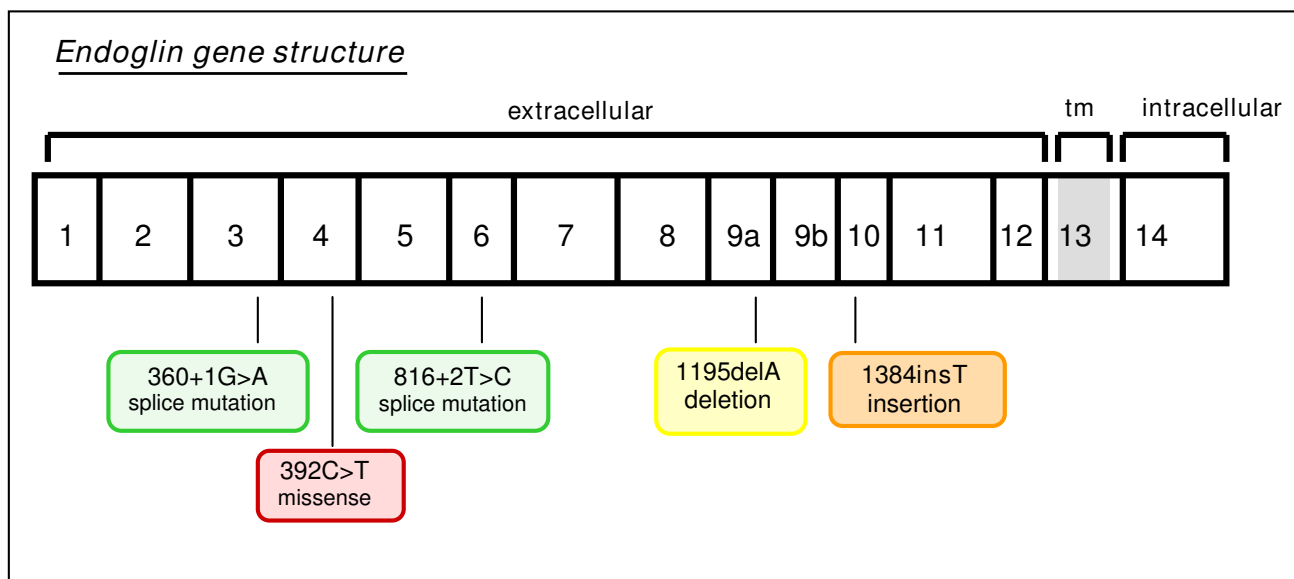


Figure 2
Distribution of mutations in the *ENG* gene. Novel mutation is given in bold.

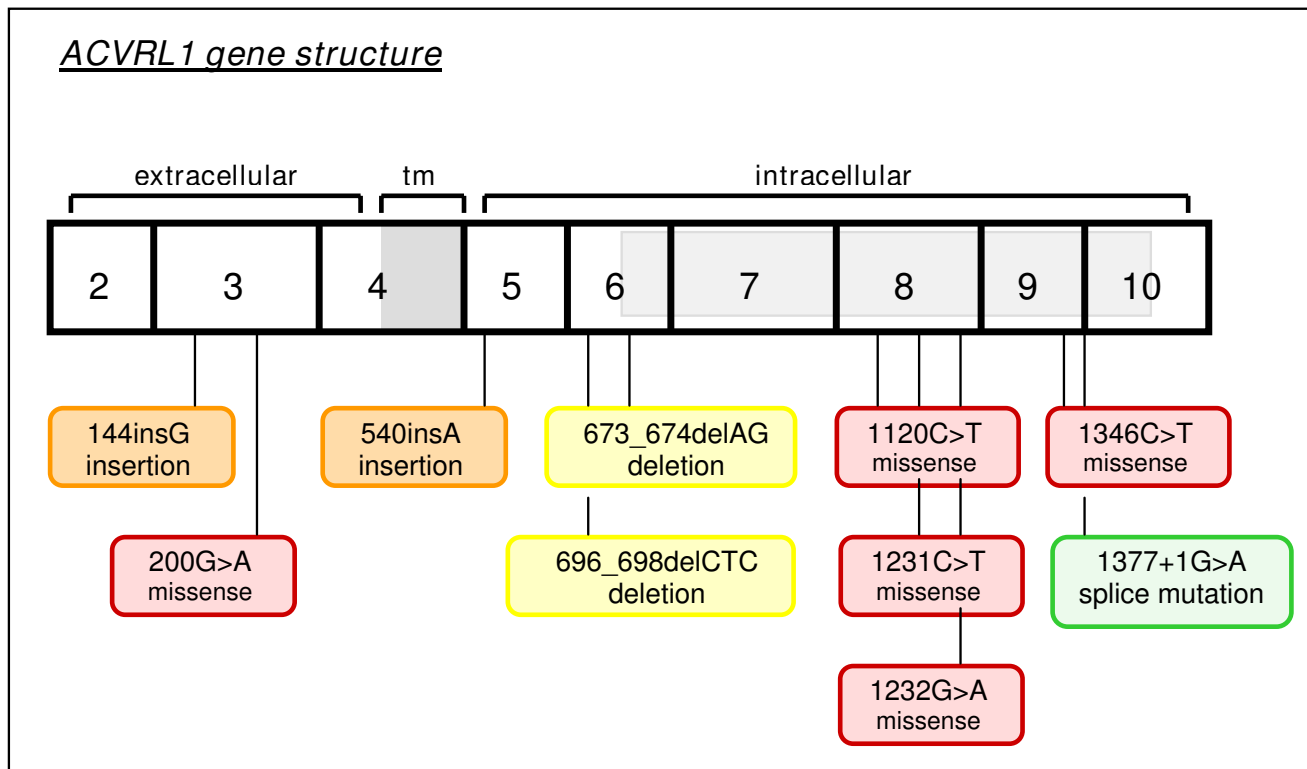


Figure 3
Distribution of mutations in the *ACVRL1* gene.

telangiectasias, 3) organ manifestation with arteriovenous malformations (AVMs) including pulmonary, cerebral, hepatic, gastrointestinal and/or 4) hereditary with a first-degree relative with HHT [13]. All patients included in this study either met the criteria for manifest clinical diagnosis or suspected clinical diagnosis of HHT with at least three or two criteria present. Altogether, 71% of the patients (29 out of 41) had a manifest HHT diagnosis. All of the patients had as main clinical symptom recurrent epistaxis and a positive family history of HHT, followed by telangiectasias in 65.8% and AVMs of any type in 56% of the cases. Of the 41 patients tested, 18 patients were found to have sequence alterations, with 6 patients showing mutations for *ENG* and 12 patients revealing mutations for *ACVRL1*. The genotype-phenotype correlation of all HHT patients with confirmed mutation is given in Additional file 7.

Discussion

Presently, *ENG* (endoglin) on chromosome 9q33-q34 and *ACVRL1* (activin-receptor-like kinase) on chromosome 12q13 are the two genes primarily implicated in the development of Hereditary Hemorrhagic Telangiectasia (HHT). In literature, frequent mutations in both genes have been detected in different case studies indicating a

common occurrence of *ENG* and *ACVRL1* DNA changes in patients with HHT [27,28].

In a consecutive series of 41 patients, we performed clinical genetic testing for the two major types of disease, HHT1 and HHT2, caused by mutations in the *ENG* and *ACVRL1* gene sequence for HHT. The series included 29 patients with clinical diagnosis of HHT who met three or more clinical features and 12 patients with suspected diagnosis with two clinical features according to the Curaçao criteria. The mutation detection rate in our series of 41 German patients was 44%. Based on the Curaçao criteria, 15 of the 18 patients (83%) with a mutation had a manifest clinical HHT diagnosis, with the remaining 3 patients (17%) fulfilling two criteria. This rate is similar to other studies which have been consistently described in literature [17,27,28]. Most of the mutations were found in the *ACVRL1* gene with a total of 10 different mutations in 12 HHT patients whereas a total of 5 mutations were identified in the coding sequence of the *ENG* gene in 6 individuals. Most of the *ENG* mutations were widely distributed throughout the gene, not indicating any special hot spot areas within the gene. Out of five detected DNA changes, four mutations had been described previously as mutations in the HHT mutation database or recently been pub-

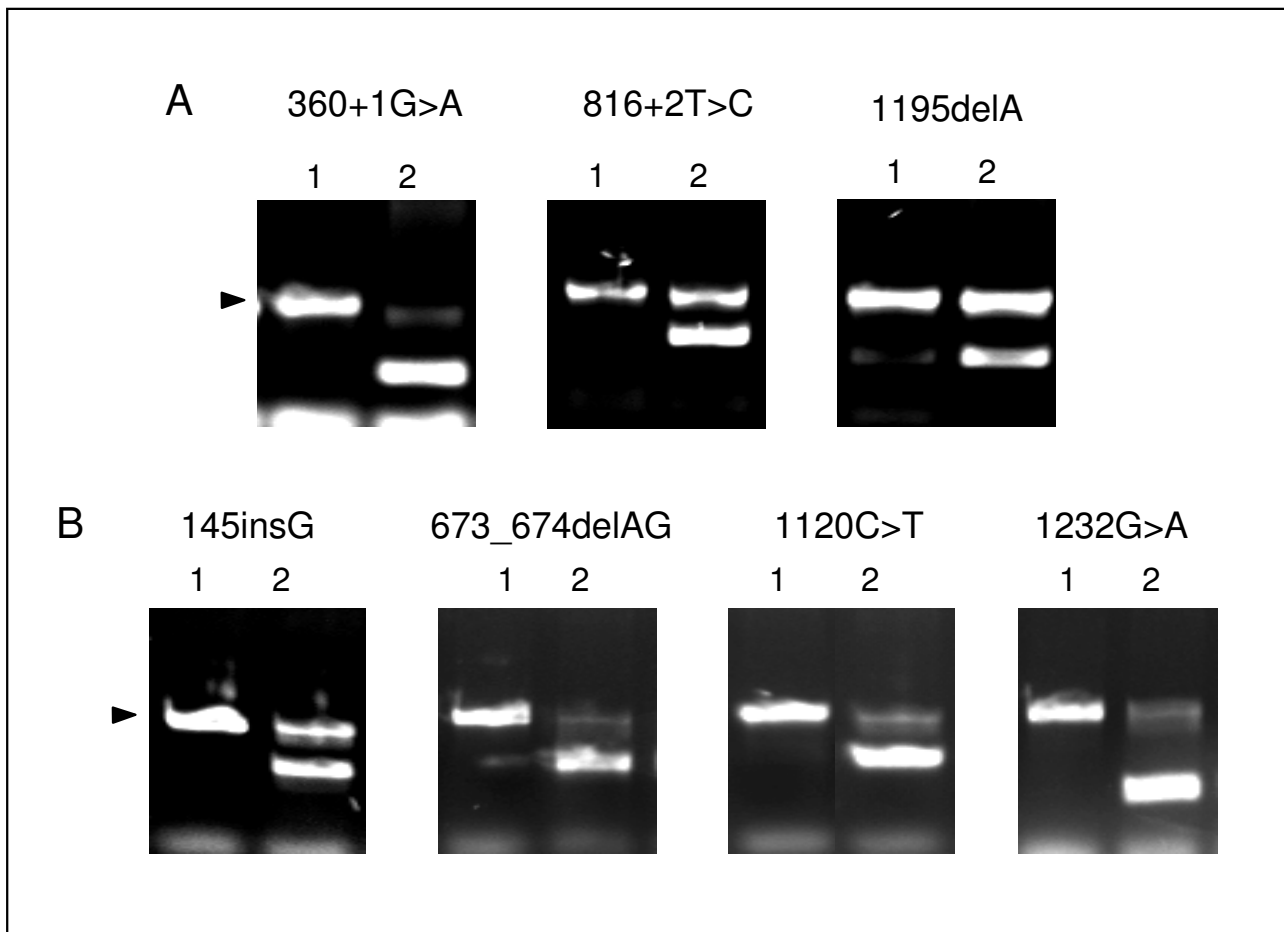


Figure 4
Representative results from PCR-SSP analysis of different *ENG* (A) and *ACVRL1* (B) mutations. Three of the 5 PCR-SSP systems for *ENG* mutations and 4 of the 10 PCR-SSP systems for *ACVRL1* mutations are shown as examples. The specificity of PCR-SSP for the corresponding mutations was given by the absence of the specific PCR product in healthy controls (each lane 1) and presence of the PCR product in the HHT patient (each lane 2). The internal control fragment of 540 bp in size (arrow head) was present in each reaction (or at least where the PCR-SSP product was absent).

lished [2,5,20,27,31-35]. The alterations included mainly nucleotide substitutions such as missense, splice site and frameshift mutations. In spite of the fact that only a small series of HHT patients was investigated in this study, one novel *ENG* mutation c.1384insT (p.Gln462fs) could be detected which had not been described before in the HHT mutation database. Unlike *ENG*, 50% of the mutations in the *ACVRL1* gene were missense substitutions. Similar results had been published by Abdalla et al. who analysed all 123 till then known mutations of *ACVRL1*. In their study overview they could also register more than half (53%) of the mutations in the *ACVRL1* gene as missense mutations [36,37]. As previously described in other HHT families, the frequency of *ACVRL1* mutations was highest in exon 8 which might be an indicator for mutation hotspots. The other mutations c.144_145insG

(p.Ala49fs), c.200G>A (p.Arg67Gln), c.540_541insA (p.Asp181fs), c.673_674delAG (p.Ser225fs), c.696_698delCTC (p.Ser233del), c.1346C>T (p.Pro449Leu) and c.1377+1G>A in *ACVRL1* were already described by other workgroups, mostly located in the intracellular kinase domain of the gene [28,35,37-40]. Only two already known mutations could be detected in the extracellular domain of the gene. However, large deletions and insertions in *ENG* and *ACVRL1* were not analyzed which could explain the low detection rate of mutations in our study compared to other studies described in literature [28,32,36].

As far as the genotype-phenotype correlation is concerned, most of the pulmonary arteriovenous malformations (PAVMs) in our study were detected in HHT patients

with mutations for *ENG*. This phenomenon has already been described by previous studies given in literature, stating that mutations of *ENG* are mainly observed in HHT type 1 with an incidence of up to 40% for PAVMs, whereas mutations of *ALK1* are primarily observed in HHT type 2 with an incidence of only 14% for PAVMs, which clinically distinguishes these two types of mutation [3,7,28,36]. Liver manifestations were more frequent in patients with *ACVRL1* mutations, whereas manifestations of the skin and the GI-tract were seen equally distributed amongst HHT patients with *ENG* and *ACVRL1* mutations.

Many studies report a mutation detection rate of up to 92% [27,28,33]. However, in our study we were able to identify only 43.9% of disease-causing mutations in a series of 41 HHT patients. The difference in sensitivity may result from several factors, including a very heterogeneous cohort of patients in terms of locations and initial physician involved, and because patients with only 2 Curaçao criteria were included in the study. In addition, only *ENG* and *ACVRL1* genes were screened for mutations, but in some cases, mutations in *SMAD4*, *HHT3* and *HHT4* loci may be responsible for the clinical symptoms [24-26].

In daily clinical routine, the diagnosis of HHT is primarily based on the clinical findings according to the Curaçao criteria. Nevertheless, in many cases clinical diagnostic is at times very difficult due to the heterogeneous outcome and manifestation of the disease. This fact asks for additional diagnostic tools which facilitate the daily clinical work. Already in the past, many studies could show that the molecular characterization of HHT enables to predict the diagnosis before the manifestation of clinical symptoms. The results of the study reflect the very high and heterogeneous distribution and allocation of mutations in HHT. With an allele-specific PCR technique (PCR-SSP) we could establish a diagnostic testing device for rapid genotyping of mutations. The exon sequencing of *ENG* and *ACVRL1* confirmed the previously described polymorphisms and outlined one novel mutation Gln462fs in exon 10 of *ENG* gene. The PCR-SSP technique can easily be performed with standard laboratory equipment and may help to minimize costs for genetic testing in the routine diagnosis of family members of HHT patients with known mutations.

Conclusion

In summary, this study is a further contribution to the determination of already known *ENG* and *ACVRL1* mutations as well as the detection of one novel *ENG* mutation in HHT. Next to the Curaçao criteria, genetic analysis certainly contributes as an essential tool to a reliable diagnosis of clinically affected HHT patients and clinically asymptomatic HHT patients, thus helping to take early pre-

ventive measures even before the occurrence of first clinical symptoms. The PCR-SSP technique could facilitate this high task of genetic analysis in routine HHT diagnostics and underlines the importance of using molecular diagnosis for early identification of individuals carrying mutations and being at risk of vascular complications.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All the authors have read and approved the final manuscript. A large part of the mutational analysis was done by HS, JH under the supervision of PB. The rest of the mutational analysis was done by UG, JS, KH completing the analysis of mutations. Bank data collection, writing of the manuscript and the direction of all the work was done under HS, the main author.

Additional material

Additional file 1

Table 1. Overview on the detailed clinical data of all HHT patients.

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[<http://www.biomedcentral.com/content/supplementary/1471-2350-10-53-S1.doc>]

Additional file 2

Table 2. Diagnostic criteria according to the Curaçao criteria for the diagnosis of HHT.

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Additional file 3

Table 3. Primers used in PCR-SSP genotyping for mutations in the *ENG* and *ACVRL1* genes.

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[<http://www.biomedcentral.com/content/supplementary/1471-2350-10-53-S3.doc>]

Additional file 4

Table 4. Summary of mutations identified in the *ENG* gene.

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[<http://www.biomedcentral.com/content/supplementary/1471-2350-10-53-S4.doc>]

Additional file 5

Table 5. Polymorphisms in *ENG*.

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[<http://www.biomedcentral.com/content/supplementary/1471-2350-10-53-S5.doc>]

Additional file 6

Table 6. Summary of mutations identified in the *ACVRL1* gene.

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[<http://www.biomedcentral.com/content/supplementary/1471-2350-10-53-S6.doc>]

Additional file 2

Table 7. Genotype-phenotype correlation in HHT patients with confirmed mutation.

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[<http://www.biomedcentral.com/content/supplementary/1471-2350-10-53-S7.doc>]

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