Novel mutations in the *ENG* and *ACVRL1* genes causing hereditary hemorrhagic teleangiectasia

LOUKAS ARGYRIOU, STEFAN TWELKEMEYER, IRAKLI PANCHULIDZE, LARS-ERIK WEHNER, UTE TESKE, WOLFGANG ENGEL and KARIM NAYERNIA

Institute of Human Genetics, Georg-August University, Heinrich-Düker-Weg 12, 37073 Göttingen, Germany

Received October 10, 2005; Accepted December 5, 2005

Abstract. Hereditary haemorrhagic teleangiectasia (HHT) is an autosomal dominantly inherited disorder characterised by cutaneous and mucosal telangiectasias, epistaxis and arteriovenous malformations in lung, liver, central nervous system and gastrointestinal tract. Mutations in the genes for endoglin *(ENG)* and for activin A receptor type II-like kinase 1 *(ACVRL1)* have been identified to cause HHT. We performed molecular diagnosis in clinically affected probands of 52 HHT families and detected mutations in 34 cases. We report on a total of 19 novel disease-causing mutations, 7 in *ENG* and 12 in *ACVRL1*. Three of the novel mutations affected acceptor splice-sites in the *ENG* gene. RNA analyses in these three patients and in two further patients described before resulted in reduction of the transcript or in a shortened transcript. Furthermore, we identified a family with the mutation c.199C>T in the *ACVRL1* gene with liver AVMs. This is the fifth family with this mutation and liver AVMs, clearly indicating a genotype-phenotype correlation for this mutation.

Introduction

Hereditary hemorrhagic telangiectasia or Osler-Weber-Rendu disease (HHT; OMIM 187300) is an autosomal dominantly inherited, genetically heterogeneous syndrome characterized primarily by epistaxis, telangiectasias and multi-organ vascular dysplasias. Its prevalence ranges from 1:39000 in Northern England (1) to 1:1331 in Netherlands Antilles (2). Clinical diagnosis of HHT is made according to the established Curaçao criteria, with definitive diagnosis of HHT being established if 3 or 4 of the criteria epistaxis, telangiectasia, visceral manifestations and family history of HHT are present. Mutations in endoglin *(ENG)* (GenBank accession no. of reference sequence: NM_000118) and activin A receptor type II-like kinase 1 *(ACVRL1)* (GenBank accession no. of reference sequence: NM_000020) cause HHT1 and HHT2, respectively. Endoglin is a TGF-ß type III receptor, while activin A receptor type II-like kinase 1 acts as a TGF-ß type I receptor. We report on a total of 19 novel disease-causing mutations in the *ENG* and *ACVRL1* genes.

Correspondence to: Dr Karim Nayernia, Institute of Human Genetics, University of Göttingen, Heinrich-Düker-Weg 12, 37073 Göttingen, Germany
E-mail: knayern@gwdg.de

Key words: Osler-Weber-Rendu, endoglin, ALK-1, mutation analysis, RT PCR

Materials and methods

Patients. Blood samples of 53 probands were sent to our institute for molecular diagnosis of HHT. Clinical data of these patients were obtained through a questionnaire by the referring physicians.

Methods. DNA was extracted from peripheral blood. All coding regions and flanking intronic sequences of the *ENG* and *ACVRL1* genes were amplified by PCR. Primer sequences and the PCR conditions are available upon request. PCR products were run on 1% agarose/0.5 x TBE gels. Purification of PCR products were performed using a PCR purification kit (Millipore, Eschborn, Germany). The purified PCR products were sequenced in forward and reverse orientation on a MegaBACE 1000 sequencer using the DYEnemic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden), according to the manufacturer's instructions. Nucleotide sequence variations were confirmed by a second PCR followed either by sequencing or by RFLP analysis. In order to confirm the relevance of all non-truncating mutations, the presence of each mutation was excluded in 50 healthy controls. The mutation nomenclature used in this study is in accordance with the rules proposed by Den Dunnen and Antonarakis (3). Blastp was used for protein alignment (http://www.ncbi.nlm.nih.gov/blast).

In order to examine the effect of splice-site mutations, we performed RT-PCR analysis in the 3 patients with splice-site mutations in the present collective and on 2 further patients previously described by Wehner *et al* (4). In all 5 cases, it dealt with splice-site acceptor mutations. The splice-site prediction program of the Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/splice.html) indicated that, in all cases, the exon 3' downstream of the mutation should be deleted. To confirm this prediction, total RNA was extracted from peripheral blood through the method described by Chetverina *et al* (5). The RNA template was then used for RT-PCR. Exonic primers for the RT-PCR were designed so

Table I. Novel mutations described in this study, their location on genomic level, their effect on protein level and symptoms of the affected patients.[a]

Mutation (cDNA)	Location	Type	Protein	Pat. no.	Sympt.
ENG					
1. c.166C>T	Ex 2	N	p.56X	24920	E,T,f
2. c.344delC	Ex 3	D	fs	24932	E,C,f
3. c.577_582delACGCTCinsGTACTCCAG	Ex 5	D+I	p.T193_L194del VLQins	24949	E,T,P,f
4. c.644delA	Ex 5	D	fs	24907	E,T,f
5. c.690-1G>A	Int 5	S	?	24924	E,f
6. c.1315_1322dupAAGGTGCA	Ex 10	Dup	fs	24938	E
7. c.1634G>A	Ex 11	M	p.G545D	24927	E,T,f
ACVRL1					
8. c.148T>G	Ex 3	M	p.W50G	24908	E,f
9. c.197A>C	Ex 3	M	p.H66P	26507	E,T,f
10. c.205T>C	Ex 3	M	p.C69R	24952	E,T,f
11. c.319delC	Ex 4	D	fs	24921	E,T,f
12. c.526-7C>G	Int 4	S	?	24967	E,T,f
13. c.526G>T	Ex 5	M	p.D176Y	24915	E,T,H
14. c.626-9_630delGGCCATCAGGAAA	Int 6-Ex 6	S	?	22587	E,T,P,f
15. c.698C>T	Ex 6	M	p.S233L	26508	E,T,P,f
16. c.793A>C	Ex 7	M	p.T265P	24995	E,P,f
17. c.1208T>C	Ex 8	M	p.L403P	24598	E,T,Sp,f
18. c.1246G>A	Ex 8	M	p.G416S	24946	H,P,f
19. c.1302_1303delCTinsA	Ex 9	D+I	fs	24933	E,T

[a]E, epistaxis; T, telangiectasia; P, pulmonary AVM; C, cerebral AVM; G, gastrointestinal AVM; H, hepatic AVM; Sp, spleen AVM; f, family history of HHT; D, deletion; I, insertion; S, splice site; N, nonsense; M, missense; fs, frameshift; Dup, duplication; Ex, exon.

as to span every exon which was expected to be deleted. In one case, a 3'RACE-PCR (6) was performed, as in the respective patient the splice-site mutation is localized before the last exon of the *ACVRL1* gene.

Results and Discussion

We analysed 52 patients with clinically definitive (3 of 4 Curaçao criteria) or suspected (2 of 4 Curaçao criteria) diagnosis of HHT and detected 34 mutations. This represents a mutation detection rate of 65.4%, which is in accordance with reports of other large screening studies (4,7). We detected 19 mutations in *ENG* and 15 in *ACVRL1*. Seven of the 19 *ENG* mutations and 12 of the 15 *ACVRL1* mutations were novel. We excluded these mutations in 50 healthy controls. The affected amino acids with the exception of the one affected by the mutation *ACVRL1* c.197A>C are highly conserved, as revealed by a protein alignment for *ENG* and *ACVRL1* in various species. A list of the novel mutations described here and the clinical data for each patient are given in Table I, the localization of the mutations in both genes is presented in Fig. 1.

The novel mutations in *ENG* are 3 frameshift mutations (c.344delC, c.644delA and c.1315_1322dupAAGGTGCA), 1 missense mutation (c.1634G>A), 1 nonsense mutation (c.166C>T), 1 in frame substitution (c.577_582delACGCTC insGTACTCCAG) and 1 splice-site mutation (c.690-1G>A). In *ACVRL1* we discovered 8 missense mutations (c.148T>G, c.197A>C, c.205T>C, c.526G>T, c.698C>T, c.793A>C, c.1208T>C and c.1246G>A), 2 frameshift mutations (c.319delC and c.1302_1303delCTinsA) and 2 splice-site mutations (c.526-7C>G and c.626-9_630delGGCCATCAG GAAA, which deletes a splice acceptor site). The duplication c.1315_1322dupAAGGTGCA in the *ENG* gene in patient 24938 is the third example for a duplication of more than one base described in the literature (7,8). The deletion c.626-9_630delGGCCATCAGGAAA is, to the best of our knowledge, the first deletion of a splice-site acceptor described in a patient with HHT.

For the 5 frameshift mutations and the nonsense mutation, no further experiments were performed, as such mutations are predicted to result in a truncated protein. Truncated and, thus, non-functional proteins are per se disease-relevant, as HHT is caused by haploinsufficiency of *ENG* or *ACVRL1* (9,10).

In the case of patient 24927 (Table I), blood samples of further affected and non-affected family members were available. The mutation in this family (ENG c.1634G>A)

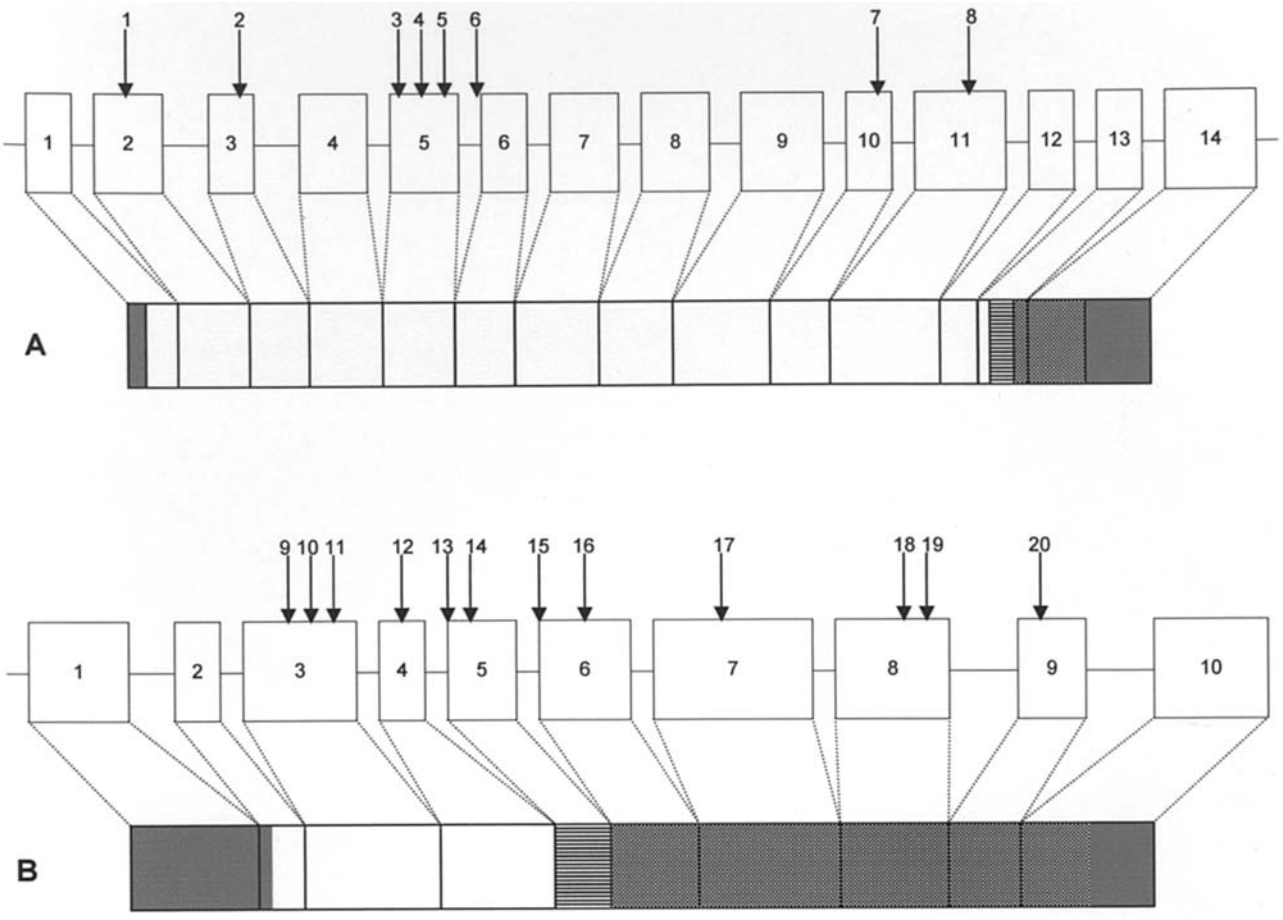

Figure 1. The mutations are spread in various exons of both genes. However, a relative accumulation of the described mutations can be observed in the exons 3-6 of the *ENG* gene. (A) Upper panel, genomic structure of endoglin gene and localisation of the novel mutations described in this study. The numbers in the boxes indicate the exons of the gene. The corresponding patients are described in Table I (the number of each mutation is given above the arrows). Lower panel, structure of endoglin protein. (B) Upper panel, genomic structure of *ACVRL1* gene and localisation of the novel mutations described in this study. The numbers in the boxes indicate the exons of the gene. The corresponding patients are described in Table I (the number of each mutation is given above the arrows). Lower panel, structure of ACVRL1 protein. Grey box, non-coding; white box, extracellular; striped box, transmembrane; hatched box, intracellular.

was found to segregate with the phenotype of HHT, further indicating that it causes HHT.

One patient has the previously described (11) mutation c.199C>T in the *ACVRL1* gene. This patient shows telangiectasias on the lips as well as massive and frequent epistaxes. Moreover, she has confirmed liver AVMs since 1993. Due to left-right shunts and beginning right heart decompensation, she has been planned for liver transplantation. Her mother and grandmother died of severe liver disease at the ages of 72 and 60, respectively. Four additional families with the same mutation and a severe liver phenotype were described in the literature (11,12). Since 5 unrelated families present the same mutation, we can assume that the mutation c.199C>T in the *ACVRL1* gene leads to a severe liver phenotype. To the best of our knowledge, this is the first HHT mutation, for which a phenotype-genotype correlation can be assumed.

In one family, we detected the sequence variant c.572G>A in the *ENG* gene. Although it seemed to segregate with the HHT phenotype in this family, Lesca *et al* (7) and Abdalla *et al* (13) reported the same variant in 6.4% of healthy controls in heterozygous state and in 1.4% in homozygous state. We also found this variant in 2% of healthy controls. Thus, we assume that it is a non-disease relevant sequence variation. Since none of the members of this family has a further variation in *ENG* or *ACVRL1*, we suspect that a mutation in another gene causes HHT in this family.

In order to investigate the possible effect of splice-site mutations on the mRNA level, we performed RT-PCR analysis in the 5 patients with splice-site mutations detected so far in our collective [3 in this study (24924, 24967 and 22587) and 2 reported by Wehner *et al* (4)]. In each of these families, the phenotype segregates with the mutation. Moreover, all of the splice-site mutations were excluded in non-affected members of the families. In all cases, the absence of one exon was predicted. However, we were able to confirm this only in the case of patient 24904. In the remaining 4 cases, only the wild-type transcript could be detected (Fig. 2). However, as seen on Fig. 2, the wild-type transcript in these patients seems to be weaker than in the healthy controls. Therefore, we assume that the aberrant transcript is instable and is degradated via a nonsense-mediated decay-like mechanism. This explanation is consistent with previously published data which support that HHT is due to a mechanism of haploinsufficiency (9,10).

Conclusively, we would like to comment that HHT patients should be examined for mutations only after detailed

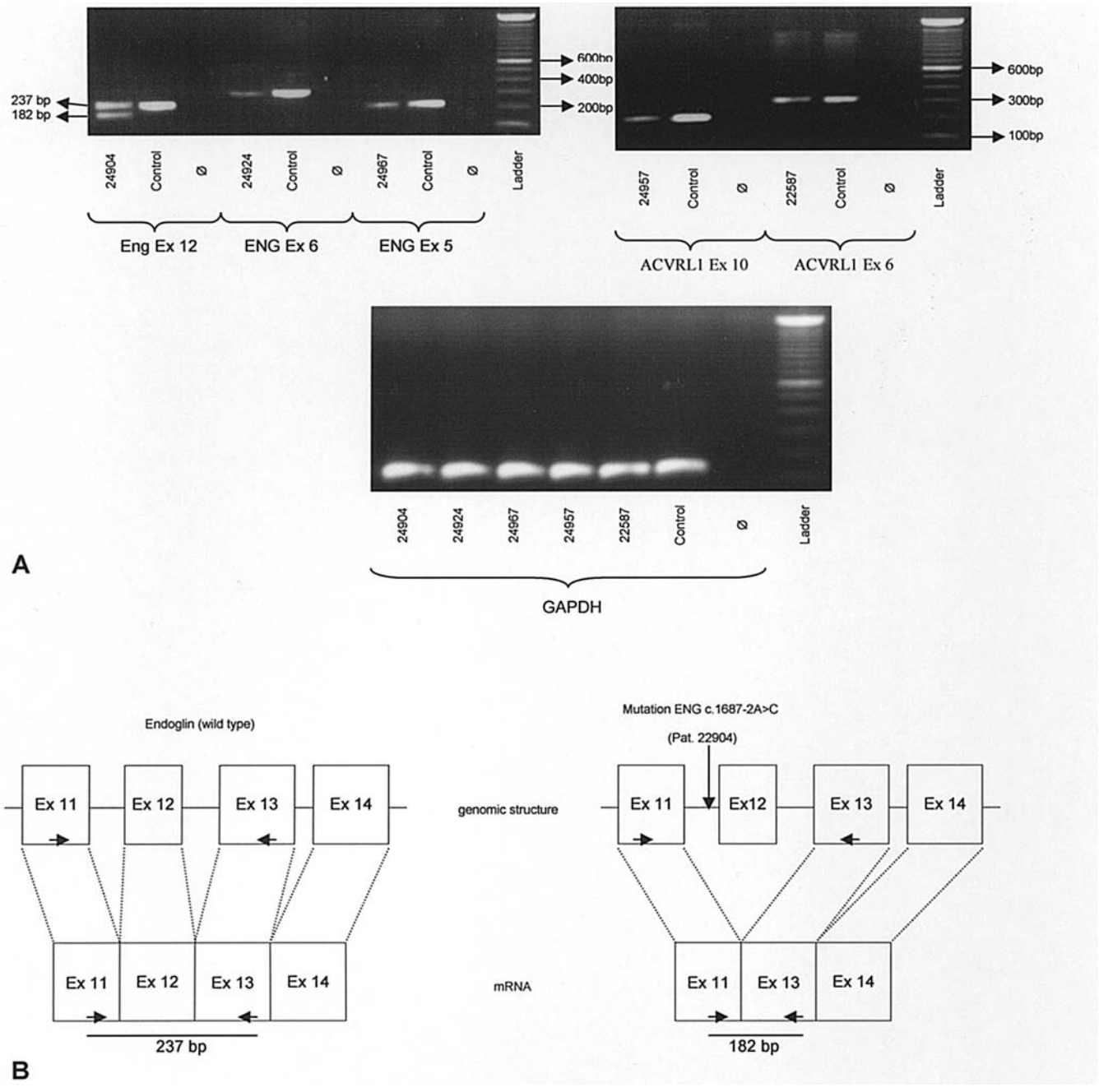

Figure 2. (A) RT-PCR of RNA from patients with splice-site mutations. A shorter transcript (182 bp) which lacks exon 12 (55 bp) can only be detected in Pat. 24904 (wild-type product in this case: 237 bp). In all the other patients, a weaker amplicon for the wild type transcript was found. (B) On the left, normal splicing of endoglin. On the right, localisation of splice-site mutation of Pat. 24904 on genomic DNA and its effect on the mRNA level. The arrows indicate the localization of the primers used for the RT-PCR.

clinical examinations. This would allow the drawing of further genotype-phenotype correlations in the future, a fact which would prove important in terms of genetic counselling and prediction of HHT symptoms.

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