Hum Genet (2005) 116: 8–16
DOI 10.1007/s00439-004-1196-5

ORIGINAL INVESTIGATION

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Hereditary hemorrhagic telangiectasia: *ENG* and *ALK-1* mutations in Dutch patients

Received: 8 June 2004 / Accepted: 7 September 2004 / Published online: 23 October 2004

Abstract Hereditary hemorrhagic telangiectasia (HHT) or Rendu-Osler-Weber disease is an autosomal dominant disorder characterized by an aberrant vascular development. The resulting vascular lesions range from smaller mucocutaneous telangiectases to large visceral arteriovenous malformations, especially in the skin, lung, gastrointestinal tract and the brain. Mutations in the genes encoding endoglin (*ENG*, chromosome 9q34) and activin A receptor type-like kinase 1 (*ALK-1*, also named *ACVRL1*, chromosome 12q13) are associated with HHT1 and HHT2, respectively. We report here on the genetic and molecular heterogeneity found in the HHT population in the Netherlands. Probands of 104 apparently unrelated families were studied and we performed sequence analysis on both the *ENG* gene and *ALK-1* gene. In most of the probands, we found a mutation in one of the two genes: 53% in the *ENG* gene and 40% in the *ALK-1* gene. In 7% of the families no *ENG* or *ALK1* mutation was found. The mutations detected were deletions, insertions, nonsense, missense and splice site mutations. The majority were novel mutations.

Introduction

Hereditary hemorrhagic telangiectasia (HHT) or Rendu-Osler-Weber (ROW) syndrome is an autosomal dominant disease characterized by vascular malformations in multiple organ systems. The clinical manifestations are caused by direct arteriovenous connections without an intervening capillary bed. This can result in a range of malformations from smaller mucocutaneous telangiectases to large visceral arteriovenous malformations (AVM). The clinical manifestations of HHT include recurrent epistaxis, multiple telangiectases, at characteristic sites (lips, oral cavity, nose, fingers), visceral lesions, such as gastrointestinal telangiectases, pulmonary arteriovenous malformations (PAVM), cerebral arteriovenous malformations (CAVM) and hepatic arteriovenous malformations (HAVM) (Guttmacher et al. 1995; Shovlin and Letarte 1999). Recurrent epistaxis is usually the first symptom and present in more then 90% of patients with HHT (Haitjema et al. 1996a; Shovlin and Letarte 1999). Telangiectases usually start to appear in the third decade of life and can be detected in more than 80% of the HHT patients (Guttmacher et al. 1995; Haitjema et al. 1996b; Shovlin and Letarte 1999). Gastrointestinal bleeding from intestinal telangiectatic lesions usually does not start before fifth decade and may cause severe anemia (Guttmacher et al. 1995; Haitjema et al. 1996b). The most common site of AVM is the lung. Pulmonary arteriovenous malformations are estimated to develop in more then 20% of the patients (Shovlin and Letarte 1999). PAVMs result in a right to left shunt and thus hypoxemia and may cause serious complications, such as bleeding (hemothorax), bypass of emboli or bacteria causing serious systemic complications (stroke, cerebral abscess) (Guttmacher et al. 1995; Shovlin and Letarte 1999). Because of these serious complications, treatment of PAVMs is indicated, even when asymptomatic. Cerebral arteriovenous malformations (CAVMs) are less common, but are probably under-recognized, and present in 10–15% of the patients (Willemse et al. 2000). Although they are often silent, they can cause headache, seizures, ischemia and bleeding (Haitjema et al. 1995).

Mutations in the endoglin (*ENG*, OMIM 131195) and activin A receptor type-like kinase 1 (*ACVRL-1* or *ALK-1*, OMIM 601284) genes are associated with HHT. In

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1994, *ENG* was identified on chromosome 9q34 as the gene, when mutated, responsible for HHT1 (OMIM 187300) (McAllister et al. 1994). In 1996, mutations in *ALK-1*, located on chromosome 12q13, were found to cause HHT2 (OMIM 600376) (Johnson et al. 1996). Both genes are expressed predominantly in endothelial cells. The proteins mediate binding and signaling of TGFβ, which is important in blood vessel growth and repair (Van den Driesche et al. 2003). Protein expression studies in human umbilical vein endothelial cells and peripheral blood monocytes have confirmed haploinsufficiency as the model in HHT1 and HHT2 (Bourdeau et al. 2000; Abdalla et al. 2003; Van den Driesche et al. 2003). Mutations in *ALK-1* and *ENG* both result in epistaxis and telangiectases, but a higher prevalence of PAVMs and CAVMs has been reported in HHT1. Families with HHT2 generally also show a later onset of the symptoms (Berg et al. 1996; Shovlin and Letarte 1999; Berg et al. 2003). However, within families there is considerable inter- and intra-familial variability with respect to age-related penetrance and pattern of clinical expression of the disease.

Recently, Gallione et al. (2004) reported mutations in the *MADH4* gene in patients that show clinical features of both HHT and juvenile polyposis. *MADH4* codes for the protein SMAD4 and is expressed in a variety of cell types. The protein has a role in the TGFβ pathway, like *ENG* and *ALK-1*, as well as in the bone-morphogenic-protein pathway. HHT as part of this syndrome can thus be explained by *MADH4* mutations.

To date, more than 111 different mutations have been identified in the *ENG* gene (Cymerman et al. 2003; Lastella et al. 2003; Lesca et al. 2004), whereas the number of different mutations reported in the *ALK-1* gene is 77 (Van den Driesche et al. 2003; Lesca et al. 2004). Almost all the mutations reported are unique for a particular family.

In this study, we report on the genetic molecular analysis of the probands of 104 families from the Netherlands with the clinical diagnosis HHT, to provide insight into the genetic and molecular heterogeneity of patients living in the Netherlands.

Materials and methods

Patients

All probands were from a single tertiary referral center in the Netherlands, specializing in the diagnosis and treatment of HHT. All but eight families were of Dutch origin; two families were from Belgium (F112, F113), one came from former Yugoslavia (F26), one from Turkey (F37), one from Iran (F36), one from Lithuania (F83), one from the Netherlands Antilles (F153) and one family from Surinam (F25).

The medical and family history of each patient was recorded. The diagnosis was established according to the Curacao criteria (Shovlin et al. 2000). At least three of the following four criteria were required for a clinical diagnosis: spontaneous and recurrent epistaxis, telangiectases at characteristic sites, visceral manifestations (PAVM, CAVM, HAVM) and a first-degree relative with HHT.

Amplification and nucleotide sequence determination

High molecular weight DNA of the probands was isolated from peripheral blood leukocytes according to established procedures. The exons 1–14 of the *ENG* gene and exons 1–10 of the *ALK-1* gene and their flanking sequences were amplified using polymerase chain reaction (PCR). The amplification primers were derived from the *ENG* genomic sequence (GenBank: BC014271.2) and *ALK-1* genomic sequence (GenBank: NM_000020.1) (see Table 1). The PCR was carried out essentially as described by Bergman et al. (1998). For exons 7 and 8 of the *ALK-1* gene, the GeneAmp 10×PCR buffer II (Applied Biosystems, Foster City, Calif., USA) was used. Amplification was performed on a Gene Amp 9700 thermal cycler (Applied Biosystems) using an initial denaturation step at 94 °C for 4 min, followed by 33 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min for the exons. For *ENG* exons 5 and 14 an annealing temperature of 58°C was used.

The amplified fragments were first analyzed by agarose-gel electrophoresis. Next, the PCR products were purified using the QIAquick 96 PCR BioRobot Kit (Qiagen, Venlo, The Netherlands). The purified fragments were sequenced, using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on an ABI Prism 3700 DNA analyzer (Applied Biosystems). The sequences were compared to the reported gene sequence using the Seqscape program (Applied Biosystems).

Results

The 104 HHT probands were screened for genetic changes in the protein encoding exons and their flanking sequences of both the *ENG* and *ALK-1* gene. Exon 1 of the *ALK-1* was analyzed only for those patients who did not show a mutation in either *ALK-1* or *ENG* protein encoding exons, as it encodes the 5′-untranslated region of the mRNA only. Sequence alterations in the *ENG* and *ALK-1* gene were detected in 97 probands. *ENG* mutations were found in 55 patients (summarized in Table 2) and *ALK-1* mutations in 42 patients (summarized in Table 3). One proband showed mutations in both the *ENG* and *ALK-1* gene and one proband had two mutations in *ALK-1*. Seven probands had no mutation in either the *ENG* gene or the *ALK-1* gene.

Mutations in the *ENG* gene

Forty different mutations were identified, 29 of these have not previously been reported.

Table 1 Nucleotide sequence of the amplification primers of *ENG* and ALK1

	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Amplicon (bp)
ENG			
Exon 1	ACTGGACACAGGATAAGGCCCAG	AATACTTGGGGCCTGGTCCGTG	214
Exon 2	CACCTTATTCTCACCTGGCCTCTT	CTGCCTTGGAGCTTCCTCTGAG	282
Exon 3	GGGTGGCACAACCTATACAAAT	CAGAGATGGACAGTAGGGACCT	294
Exon 4	TTCCTGACCTCCTACATGGG	TTCAGCTCAGCAGCAGAGG	310
Exon 5	TGAGGGAAGGGACTGAGGTG	GTGGGGACTAGTGTCAGGGGC	272
Exon 6	GGCCTGTCCGCTTCAGTGTT	GTTTTGTGTCCCGGGAGCTG	237
Exon 7	CCCCCTGTTCTGCCTCTCTC	CTGATCCAAGGGAGGGGAAG	292
Exon 8	ACACATATCACACAGTGACCAGC	CTAGGGGAGGAACCAGATGTC	286
Exon 9a	CTCCTGATGGTGCCCCTCTCTT	TTGTCTTGTGTTCTGAGCCCCTG	330
Exon 9b	ATTGGGTGGGATACCCTCTGGG	GGGTTAAGCACGTGACTGTCC	247
Exon 10	ATTGACCAAGTCTCCCTCCC	GAAAGGCGGAGAGGAAGTTC	301
Exon 11	GGTGGGGTGAAGAGCAGCTG	GACCTGGAAGCTCCCACTTGAA	390
Exon 12	GAGTAAACCTGGAAGCCGC	GCCACTAGAACAAACCCGAG	188
Exon 13	CTCCTGATGGTGCCCCTCTCTT	CTCAGAGGCTTCACCTGGGCTCC	289
Exon 14	AGGACCCTGACCTCCGCC	CTCTCCTGCTGGGCGAGC	232
ALK-1			
Exon 1	AGTCGGCGAGCTGGGCAATA	ACTGAGCCTCCCGCGACCAC	375
Exon 2	CTCTGTGATTTCCTCTGGGCA	TACATTCTCCCCAGCTTCTCAA	300
Exon 3	AGCTGGGACCACAGTGGCTGA	GGAGGCAGGGGCCAAGAAGAT	380
Exon 4	AGCTGACCTAGTGGAAGCTGA	CTGATTCTGCAGTTCCTATCTG	352
Exon 5	AGGAGCTTGCAGTGACCCAGCA	ATGAGAGCCCTTGGTCCTCATCCA	276
Exon 6	AGGCAGCGCAGCATCAAGAT	AAACTTGAGCCCTGAGTGCAG	330
Exon 7	TGACGACTCCAGCCTCCCTTAG	CAAGCTCCGCCCACCTGTGAA	423
Exon 8	AGGTTTGGGAGAGGGGCAGGAGT	GGCTCCACAGGCTGATTCCCCTT	327
Exon 9	TCCTCTGGGTGGTATTGGGCCTC	CAGAAATCCCAGCCGTGAGCCAC	290
Exon 10	TCTCCTCTGCACCTCTCTCCCAA	CTACCTCTACCCAGATAGGG	283

Eight different *nonsense mutations* were found. One new nonsense mutation was found in three probands (F2, F19, F49): a c.247C > T transition that results in a premature stop at codon 83 (p.Q83X). The other seven nonsense mutations were unique, six of which were novel (p.Q48X, p.C53X, p.Q360X, p.Y537X, p.Q560X, and p.Q562X). The p.R93X of F15 was reported by Cymerman et al. (2000).

Nine different *missense mutations* were found in 16 probands. Of these, the amino acid change p.W261R caused by c.781T > C was found in eight probands. In one of these probands (F68) a mutation was detected also in the *ALK-1* gene, p.A482V (c.1445C > T). The substitutions p.L306P, p.G413V, p.G191D and p.P131L have already been published (Gallione et al. 1998; Lux et al. 2000; Cymerman et al. 2003; Lesca et al. 2004). F7 showed an alteration of the very first base pair of the first codon. This alteration causes a p.M1V missense mutation and destroys the AUG initiation site of translation. In F186, the last base pair in exon 7 is changed (c.991G > A), thereby disrupting the donor splice site consensus as well. The p.K374S (F154) is caused by a substitution of 2 bp. The mutation in F145 (p.D264N) changes aspartic acid into asparagine, amino acids with different polarities. In F81 a c.572G > A resulted in a p.G191D substitution. This mutation has also been identified in a panel of normal individuals, albeit at a low frequency. It therefore suggests that the p.G191D substitution is not a disease-causing mutation.

Eight deletions, seven insertions and one complex rearrangement were identified in 18 patients. The in-frame deletion (c.787_789delATC) was found in two families and reported earlier in a French patient (Lesca et al. 2004). The c.1346_1347delCT was published earlier (Lesca et al. 2004) as well. A complex inframe rearrangement occurred in F9 and F149, which has been reported as a deletion of 21 bp by others (McAllistar et al. 1994). The rearrangement involves a 32-bp deletion and an insertion-duplication of 11 bp (see Fig. 1).

Seven *different splice site mutations* were found in 11 families. Three mutations (c.360 + 1 g > a, c.689 + 2 t > c and c.67 + 1 g > a) have been found also by others (Pece et al. 1997; Gallione et al. 2000; Cymerman et al. 2003; Lesca et al. 2004). In five probands (F43, F48, F56, F73, and F142), a p.R437R silent mutation due to a c.1311G > A transition was identified, in one proband (F45) a p.A378A due to c.1134G > A. However, both transitions occur at the last position of the exons (exon 9b and exon 8). These positions are part of the consensus sequence of the donor splice site and are highly conserved. The c.1310delG mutation identified in F16 is in the last triplet of exon 9 and will disrupt the reading frame but might also influence the consensus sequence of the donor splice site. Finally, the mutation in F151 (c.360 + 5 g > a) also changes the donor splice site creating a less favorable splice site.

Mutations in the *ALK-1* gene

Thirty-one different mutations were detected in the *ALK-1* gene, 25 of which were novel.

Seven *nonsense mutations* were found, five of which (p.W50X, p.Q118X, p.Q147X, p.Q321X, p.E470X) were

Table 2 Summary of the identified *ENG* mutations

Mutation type	Proband	Exon	Mutations cDNA	Protein	Reference
Nonsense	F112	2	c.142C > T	p.Q48X	
	F75	2	c.157C > A	p.C53X	
	F2, F19, F49	3	c.247C > T	p.Q83X	
	F15	3	c.277C > T	p.R93X	Cymerman et al. (2000)
	F10	8	c.1078C > T	p.Q360X	
	F18	11	c.1611C > A	p.Y537X	
	F54	11	c.1778C > T	p.Q560X	
	F144	11	c.1684C > T	p.Q562X	
Deletion/ insertion	F3	3	c.263delA	p.N88fs	
	F183	3	c.332_338delCCCTGGG	p.A111fs	
	F11	4	c.497_498insC	p.Q166fs	
	F4	5	c.577_578insGC	p.T193fs	
	F150	6	c.701_702insACGG	p.V234fs	
	F78	6	c.733delG	p.G245fs	
	F113	6	c.766_767insC	p.P256fs	
	F39, F131	6	c.787_789delATC	p.Idel263	Lesca et al. (2004)
	F9, F149	7	c.887_918del;c919_920ins Del32bp,ins11bp CAAGCTCCCAG		McAllistar et al. (1994)
	F36	8	c.995delG	p.G332fs	
	F55	8	c.1117_1118insT	p.K373fs	
	F77	9	c.1142_1143insT	p.K381fs	
	F28	9	c.1255delA	p.S419fs	
	F30	10	c.1317_1318insA	p.V440fs	
	F12	10	c.1346_1347delCT	p.S449fs	Lesca et al. (2004)
	F26	11	c.1437_1438delGT	p.V479fs	
Splice site	F153	1	c.67 + 1 g > a		Gallione et al. (2000)
	F37	3	c.360 + 1 g > a		Pece et al. (1997); Cymerman et al. (2003)
	F151	3	c.360 + 5 g > a		
	F6	5	c.689 + 2 t > c		Lesca et al. (2004)
	F45	8	c.1134G > A TGCGgtaa	p.A378A	
	F16	9b	c.1310delG		
	F43, F48, F56, F73, F142	9b	c.1311G > A AGCGGggag	p.R437R	
Missense	F7	1	c.1A > G	p.M1V	
	F25	4	c.392C > T	p.P131L	Cymerman et al. (2003)
	F81	5	c.572G > A	p.G191D	Lesca et al. (2004); polymorphism?
	F8, F23, F41, F59, F62, F68, F74, F176	6	c.781T > C	p.W261R	
	F145	6	c.790G > A	p.D264N	
	F14	7	c.917T > C	p.L306P	Gallioni et al. (1998)
	F186	7	c.991G > A	p.G331S	
	F154	8	c.1121_1122AA > GC	p.K374S	
	F13	9a	c.1238G > T	p.G413V	Lux et al. (2000)

novel. The c.858C > A change (F24) resulting in a p.Y286X and the c.1435C > T resulting in a p.R479X were reported earlier (Olivieri et al. 2002; Lesca et al. 2004). Nine different *deletions and two insertions* were found in 16 patients. The c.1042delG deletion was identified in seven families (F33, F44, F46, F82, F95, F123 and 152). The mutation in F42 deletes 18 bp, leaving the reading frame in tact, but deleting six amino acids at the protein level. One family carried a c.625_626insTG insertion of 2 bp next to the last coding base pair of exon 5. This mutation disrupts the reading frame but might also influence the donor splice site. In F108 a 14-bp deletion was identified that encompasses the acceptor splice site and the first nucleotides of exon 6.

Thirteen different *missense mutations* were detected in 17 patients. We identified the c.1120C > T that causes a p.R374W substitution (also reported by Berg et al. 1997; Kjeldsen et al. 2001; Abdalla et al. 2003) in three patients (F58, F83, F85). The c.1121G > A mutation (p.R374Q) (Abdalla et al. 2003; Lesca et al. 2004), the c.1231C > T (p.R411W) (Trembath et al. 2001; Abdalla et al. 2003; Lesca et al. 2004) and the c.1450C > T (p.R484W) (Trembath et al. 2001; Lesca et al. 2004) were reported earlier. The p.G309S, p.V380G, p.W406C, p.P433S, p.M438T, p.P424L, p.D330N and p.D397N mutations were all novel. Three amino-acid substitutions in the same codons were reported by others [p.D397G by Lesca et al. (2004); p.P424T by Berg et al. (1997) and p.D330Y by Olivieri et al. (2002)].The novel c.1048G > C (p.G350R) mutation involves the last base pair of exon 7, changing the donor splice site as well. In F181 two mutations were present (p.P424L and p.A482V).

Table 3 Summary of the identified *ALK-1* mutations

Mutation type	Patient	Exon	Mutation cDNA	Protein	Reference
Nonsense	F139	3	c.150G > A	p.W50X	
	F51	4	c.352C > T	p.Q118X	
	F50	4	c.439C > T	p.Q147X	
	F24	7	c.858C > A	p.Y286X	Olivieri et al. (2002)
	F34	7	c.961C > T	p.Q321X	
	F22, F141	10	c.1408G > T	p.E470X	
	F1	10	c.1435C > T	p.R479X	Lesca et al. (2004)
Deletion/ insertions	F96	3	c.83delG	p.R28fs	
	F107	3	c.190delC	p.Q64fs	
	F71	3	c.203delG	p.G68fs	
	F32	4	c.372_373insCC	p.G124fs	
	F57	5	c.625_626insTG	p.G209fs	
	F123, F44, F95, F82, F46, F33, F152	7	c.1042delG	p.D348fs	
	F137	8	c.1061_1068del8bp	p.M354fs	
	F79	8	c.1071delG	p.Q357fs	
	F5	8	c.1107_1108delAG	p.R369fs	
	F42	8	c.1120_1137del18bp	p.R374_E379del	
Splice site	F108		c.626-6del14bp		
Missense	F116	7	c.925G > A	p.G309S	
	F67, F69	7	c.988G > A	p.D330N	
	F105	7	c.1048G > C	p.G350R	
	F58, F83, F85	8	c.1120C > T	p.R374W	Berg et al. (1997); Kjeldsen et al. (2001); Abdalla et al. (2003)
	F35	8	c.1121G > A	p.R374Q	Abdalla et al. (2003); Lesca et al. (2004)
	F61	8	c.1139T > G	p.V380G	
	F53	8	c.1189G > A	p.D397N	
	F109	8	c.1218G > C	p.W406C	
	F84, F80	8	c.1231C > T	p.R411W	Trembath et al. (2001); Abdalla et al. (2003); Lesca et al. (2004)
	F181	9	c.1271C > T	p.P424L	
	F86	9	c.1297C > T	p.P433S	
	F187	9	c.1313T > C	p.M438T	
	F20	10	c.1450C > T	p.R484W	Trembath et al. (2001); Lesca et al. (2004)

Discussion

We describe here mutations in *ENG* and *ALK-1* in a large group of HHT patients living in the Netherlands. The mutations are distributed along both genes and comprise all types of mutations, e.g. affecting splice sites, missense mutations, nonsense mutations, insertions and deletions. The majority of the mutations detected were novel, since only 17 of the 71 mutations had been published earlier. Our study therefore adds 54 novel muta-

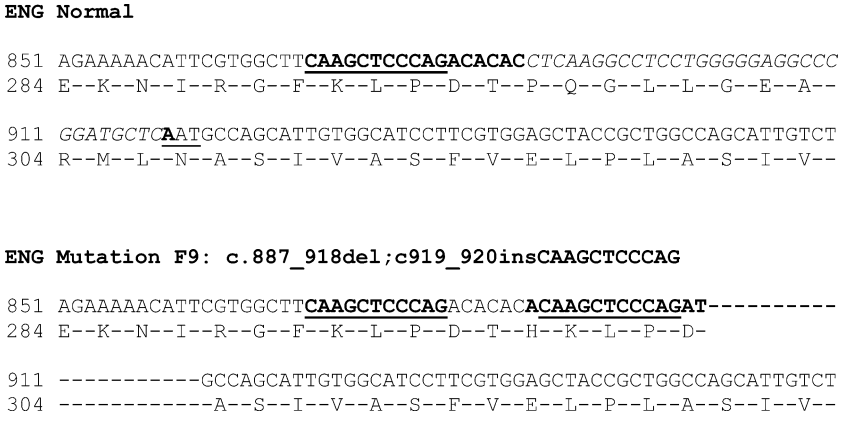

Fig. 1 The complex alteration in exon 7 of the *ENG* gene in family F9. The deleted sequence is depicted in *italics*; the duplicated sequence is *underlined in bold.*

tions to the HHT mutation database (http://genetics.mc.duke.edu/hht/). To date, 188 different HHT mutations have been described.

In the HHT families, we identified more defects in the *ENG* gene (40 different mutations, 53% of probands) than in the *ALK-1* gene (23 different mutations, 40% of probands). Ten mutations were found in more than one proband. The families were first thought to be unrelated but recent genealogical analysis has provided insight into the possibility of shared ancestry. Families with the *ENG* mutation p.W261R had a common ancestor dating back to 1765. The *ALK-1* mutation c.1042delG appears to be a founder mutation, originating from a common ancestor in 1722. Finally, all five families with the p.R437R *ENG* mutation could be traced back to a single founder in 1745.

Two probands had two mutations. Proband F181 showed two mutations in the *ALK-1* gene, a p.P424L and a p.A482V (c.1445C > T) missense mutation. It still has to be established whether they are in *cis* or in *trans*. The p.A482V was also found in proband F68 with the deleterious *ENG* mutation p.W261R, while p.A482V was also reported by D'Abronzo et al. (1999). This mutation was found in just one of 64 patients screened for *ALK-1* mutations with a pituitary tumor. The patient and his family showed no symptoms of HHT, which therefore suggests that this mutation is not the cause of HHT. However, Lesca et al. (2004) did identify this mutation in a patient with confirmed HHT. Furthermore, the alanine in this position is a highly conserved (see Fig. 2). Apparently the mutation does not always result in HHT symptoms. Functional studies should elucidate whether this mutation has a deleterious effect.

In the *ENG* gene we detected eight nonsense, eight deletions, seven insertions, one complex rearrangement, seven splice site mutations and nine missense mutations. All the insertions and all but one deletion lead to a shift in the reading frame and will result in a truncated protein that is deleterious. In addition, it has been well established that mutations involving a premature translation stop also may cause a nonsense-mediated decay of mRNA. The c.360 + 1 g > a donor splice site mutation seems to be a recurrent mutation. We found it in one family, others have found the mutation in different, independent families (Pece et al. 1997; Cymerman et al. 2003). It has also been found as a de novo mutation in one family (Cymerman et al. 2003). The p.R437R silent mutation found in F43, F48, F56, F142 and F73 is due to a c.1311G > A transversion at the last nucleotide of exon 9b. It creates a less favorable splice donor site, as confirmed by the Berkeley Drosophila Genome Project Splice Site Prediction Program. According to this program, the donor-motif probability decreased from 0.6 to undetectable. Consequently, it will hamper the proper maturation of the pre-mRNA by intron retention and/or exon skipping. Gallione et al. (1998) described a c.1311G > C change at the same position; this mutation was seen to co-segregate with the disease in the family. The donor-motif probabilities for the other splice site mutations were also unfavorable according to the prediction program.

Nine different *ENG* missense mutations were detected, one (p.W261R) was found in eight probands. The p.L306P was published by Gallione et al. (1998) as completely disrupting the helix structure. The p.G413V was described as a mutation originating from the Netherlands and present in the Dutch Antilles (Lux et al. 2000). The p.P131L was also reported by Cymerman et al. (2003). It is located in a sequence that is strongly conserved between man, mouse and pig. The mutation associates with a significantly reduced level of endoglin. The p.M1V mutation is a mutation at the very first nucleotide changing an A > G, changing the AUG start codon. A similar mutation (p.M1Y) was described by Gallione et al. (1998). These mutations are considered as disease causing by eliminating the proper start site of translation. The mutation in F186 (c.991G > A) changes the last base pair of exon 7, disrupting the donor splice site as well. The c.790G > A (p.D264N) and c.1121_1122AA > GC (p.K374S) involve amino acids that are conserved between man, mouse and pig.

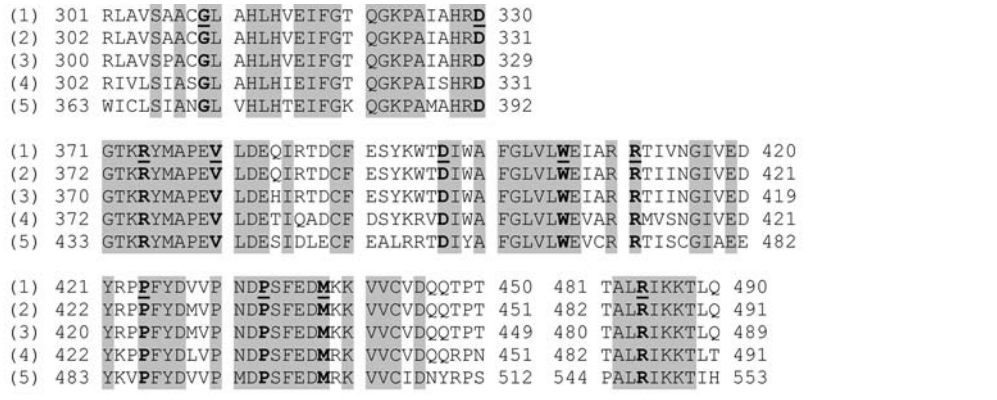

Fig. 2 Alignment of amino-acid sequence for genes orthologous to *ALK-1*.

(1) Homo sapiens, (2) Rattus norvegicus, (3) Musculus musculus, (4) Gallus gallus, (5) Drosophila melanogaster. Aminoacid changes reported in table 3 are given in bold.

The status of the p.G191D missense mutation is not obvious. The p.G191D mutation was found in two families. In one of our largest HHT families (F49), this mutation was detected in a branch of the family where the diagnosis of HHT was questionable. In another branch of this family, HHT segregated with the *ENG* nonsense mutation p.Q83X. Furthermore, the p.G191D mutation was also present at low frequency (1–5%) in a panel of normal individuals (presented at the Bonaire Annual HHT Meeting). And recently, this mutation was described in a French patient as a polymorphism (Lesca et al. 2004), but not detected in 188 French control individuals. Still, our F81 showed no other abnormality than p.G191D. This could indicate that the disease-causing mutation has not been identified so far or that p.G191D is a mutation with a reduced risk or interacts with another gene mutation not yet discovered. Functional analysis should shed light on the effect of p.G191D and other mutations.

In *ALK-1* we found seven nonsense, eight deletions, two insertion, one splice site mutation and 13 missense mutations. Exon 1 was not included in the analysis of all patients, but limited to those who did not show a mutation. Exon 1 contains no protein coding information, a deleterious mutation like a donor splice site mutation however, might be present. To date, no mutations in exon 1 of the *ALK-1* have been reported. We did not identify any mutations in exon 1 of the *ALK-1* gene in our probands.

All 13 missense mutations that we identified were located in exons 7, 8, 9 and 10. The substitutions cause either an alteration in polarity, hydrophobicity or side chain-length. Four of these missense mutations (p.R374Q, p.R374W, p.R411W and p.R484W) have already been published. Abdalla et al. (2003) reported several missense mutations in the ALK-1 cytoplasmatic domain. The amino-acid changes occurred at positions that are highly conserved between ALK-1 and ALK-5, but also between ALK-1 and other serine-threonine protein kinases. In addition, the missense mutations p.G309S, p.P424L, p.D330N, p.G350R, p.V380G, p.D397N, p.W406C, p.P433S, and p.M438T are located in the cytoplasmatic kinase domains and all involve residues conserved between ALK-1 and ALK-5. Finally, all missense mutations occurred at highly conserved residues between species (*Rattus norvegicus*, *Musculus musculus*, *Gallus gallus* and *Drosophila melanogaster*) as shown in Fig. 2. The conservation of amino acids between orthologous and paralogous sequences was analyzed using a web-based program (http://blocks.thcrc.org/sift), which predicts the effect of a missense mutation. All the codons containing missense mutations were indeed highly conserved and the mutations were predicted to disrupt the protein function. We therefore consider them as deleterious.

Before this study, no mutations had been detected in exon 5 of the *ALK-1* gene. This led to the speculation by Lesca's group that some missense mutations in exon 5 lead to a more severe phenotype, different to HHT and probably lethal. We report a frameshift mutation in exon 5 of the *ALK-1* gene, a 2-bp deletion, proving that mutations in exon 5 do occur.

Our PCR and sequence-based approach enabled the identification of more than 90% of the disease-causing mutations in a panel of 104 HHT families. We did not screen for large deletions or insertions in *ALK-1* or *ENG*, nor did we search for mutations in the promoter region or in intronic sequences apart from the splice sites. In general, large deletions may account for 5–10% of the genetic defects of monogenic disorders. We therefore plan to screen the remaining seven probands for large deletions by employing multiplex ligation-dependent probe amplification (MLPA). Alternatively, more extensive locus heterogeneity may play a role. A few families have been described that show no significant linkage to either *ENG* or *ALK-1* (Piantanida et al. 1996; Buscarini et al. 1997; Wallace and Shovlin 2000). After re-evaluating all the members in the family studied by Piantanida and Buscarini, the evidence for exclusion of chromosome 12 was no longer considered significant and analysis revealed an *ALK-1* mutation (p.R67W) (Olivieri et al. 2002). However, the presence of a third locus cannot be ruled out. In this respect, the finding of *MADH4* mutations in families with both juvenile polyposis and HHT renders it a good candidate gene.

Our observation that in an uniformly and well-classified panel of HHT families, disease-causing mutations in *ENG* or *ALK-1* were identified in more than 90% of the HHT families, taken together with the known short comings of our approach, make the existence of a prominent third HHT locus in our population less likely. We expect to be able to increase the detection rate by applying MLPA. The third HHT gene will account for only a small minority of our HHT population.

Recently, Lesca's group published molecular analyses of 160 French HHT patients and found germline mutations in 100 patients (62.5%). In a subgroup of their probands (only confirmed diagnosis), the mutation rate was 68%. They used a different DNA analysis technique in 133 patients of the 160 patients, based on heteroduplex analysis of coding regions and intronic borders after PCR amplification and electrophoresis. Because of the reduced sensitivity of the heteroduplex analysis, this might in part explain the difference in mutation detection percentage. An additional explanation is that not all their probands had a definite diagnosis of HHT.

Many mutations have been described in families with HHT. The majority of the mutations are unique; only a few mutations occur more than once. As far as has been investigated in the Dutch population (three mutations: in the c.1042delG five of the seven families had a common ancestor, in the p.W261R three families and in the p.R437R all families had a common ancestor), the mutations originate from a common ancestor. Mutations in our HHT population already published by others could also be due to these old ancestral mutations.

In our HHT panel we found more different mutations in the *ENG* gene (40) than in the *ALK-1* gene (31). The most obvious explanation is that the *ENG* gene has a correspondingly higher content of coding information (658 amino acids, 15 exons) than the *ALK-1* gene (504 amino acids, 10 exons), and consequently has more positions prone to mutation. Indeed the relative content of the coding information between *ENG* and *ALK-1* is in good concordance with the number of mutations found in the *ENG* and *ALK-1* gene.

Another explanation from a clinical point of view might be that the HHT2 phenotype is milder compared with HHT1, as suggested by Shovlin et al. (1996) and Berg et al. (2003). In HHT2, symptoms are reported to arise later and the PAVM and CAVM are less frequent. HHT1 patients are therefore likely to be more easily diagnosed and thus over-represented in the patient population we have studied.

In the French population, *ENG* mutations were found in 36 probands, and *ALK-1* mutations in 64 probands. The higher proportion of *ALK-1* mutations was partly explained by the finding of a recurrent mutation in the *ALK-1* gene, the frameshift mutation c.1112_1113dupG was found in 17 probands with a common haplotype, suggesting a founder mutation. Still, more mutations were discovered in the *ALK-1* gene than in the *ENG* gene (36 versus 34). Whether this is a population effect or due to a patient selection bias remains to be elucidated.

To conclude, our findings raise the total number of mutations in the *ENG* and the *ALK-1* genes associated with HHT significantly. The total number of published *ENG* mutations (excluding the p.G191D) is now 140 and for *ALK-1* it is 102. The mutations detected in our cohort of HHT patients offer an additional means of identifying carriers in HHT families unambiguously. In this way, appropriate medical attention can be given to only those carrying a mutation and we can prevent unnecessary screening of non-carrier children in the family.

The identification of the disease-causing mutations in a large panel of HHT families provides us with a good basis for studying the phenotype–genotype relationship for both *ENG* and *ALK-1* within and between families in detail. In this way we hope to gain more insight into the variability of clinical expression and the factors contributing to HHT.

Acknowledgements We thank Jackie Senior for carefully reviewing the article.

References

Abdalla SA, Cymerman U, Johnson RM, Deber CM, Letarte M (2003) Disease-associated mutations in conserved residues of ALK-1 kinase domain. Eur J Hum Genet 11:279–287

Berg JN, Guttmacher AE, Marchuk DA, Porteous ME (1996) Clinical heterogeneity in hereditary haemorrhagic telangiectasia: are pulmonary arteriovenous malformations more common in families linked to endoglin? J Med Genet 33:256–257

Berg JN, Gallione CJ, Stenzel TT, Johnson DW, Allen WP, Schwartz CE, Jackson CE, Porteous ME, Marchuk DA (1997) The activin receptor-like kinase 1 gene: genomic structure and mutations in hereditary hemorrhagic telangiectasia type 2. Am J Hum Genet 61:60–67

Berg J, Porteous M, Reinhardt D, Gallione C, Holloway S, Umasunthar T, Lux A, McKinnon W, Marchuk D, Guttmacher A (2003) Hereditary haemorrhagic telangiectasia: a questionnaire based study to delineate the different phenotypes caused by endoglin and ALK-1 mutations. J Med Genet 40:585–590

Bergman AJ, van den Berg IE, Brink W, Poll-The BT, Ploos van Amstel JK, Berger R (1998) Spectrum of mutations in the fumarylacetoacetate hydrolase gene of tyrosinemia type 1 patients in northwestern Europe and Mediterranean countries. Hum Mutat 12:19–26

Bourdeau A, Cymerman U, Paquet ME, Meschino W, McKinnon WC, Guttmacher AE, Becker L, Letarte M (2000) Endoglin expression is reduced in normal vessels but still detectable in arteriovenous malformations of patients with hereditary hemorrhagic telangiectasia type 1. Am J Pathol 156:911–923

Buscarini E, Buscarini L, Danesino C, Piantanida M, Civardi G, Quaretti P, Rossi S, Di Stasi M, Silva M (1997) Hepatic vascular malformations in hereditary hemorrhagic telangiectasia: Doppler sonographic screening in a large family. J Hepatol 26:111–118

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

Cymerman U, Vera S, Karabegovic A, Abdalla S, Letarte M (2003) Characterization of 17 novel endoglin mutations associated with hereditary hemorrhagic telangiectasia. Hum Mutat 21:482–492

D'Abronzo FH, Swearingen B, Klibanski A, Alexander JM (1999) Mutational analysis of activin/transforming growth factor-beta type I and type II receptor kinases in human pituitary tumors. J Clin Endocrinol Metab 84:1716–1721

Gallione CJ, Klaus DJ, Yeh EY, Stenzel TT, Xue Y, Anthony KB, McAllister KA, Baldwin MA, Berg JN, Lux A, Smith JD, Vary CP, Craigen WJ, Westermann CJ, Warner ML, Miller YE, Jackson CE, Guttmacher AE, Marchuk DA (1998) Mutation and expression analysis of the endoglin gene in hereditary hemorrhagic telangiectasia reveals null alleles. Hum Mutat 11:286–294

Gallione CJ, Scheessele EA, Reinhardt D, Duits AJ, Berg JN, Westermann CJ, Marchuk DA (2000) Two common endoglin mutations in families with hereditary hemorrhagic telangiectasia in the Netherlands Antilles: evidence for a founder effect. Hum Genet 107:40–44

Gallione CJ, Repetto GM, Legius E, Rustgi AK, Schelley SL, Tejpar S, Mitchell G, Drouin E, Westermann CJ, Marchuk DA (2004) A combined syndrome of juvenile polyposis and hereditary haemorrhagic telangiectasia associated with mutations in *MADH4* (*SMAD4*). The Lancet 363:852–859

Guttmacher AE, Marchuk DA, White RI Jr (1995) Hereditary hemorrhagic telangiectasia. N Engl J Med 333:918–924

Haitjema T, Disch F, Overtoom TT, Westermann CJ, Lammers JW (1995) Screening family members of patients with hereditary hemorrhagic telangiectasia. Am J Med 99:519–524

Haitjema T, Balder W, Disch FJ, Westermann CJ (1996a) Epistaxis in hereditary haemorrhagic telangiectasia. Rhinology 34:176–178

Haitjema T, Westermann CJ, Overtoom TT, Timmer R, Disch F, Mauser H, Lammers JW (1996b) Hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu disease): new insights in pathogenesis, complications, and treatment. Arch Intern Med 156:714–719

Johnson DW, Berg JN, Baldwin MA, Gallione CJ, Marondel I, Yoon SJ, Stenzel TT, Speer M, Pericak-Vance MA, Diamond A, Guttmacher AE, Jackson CE, Attisano L, Kucherlapati R, Porteous ME, Marchuk DA (1996) Mutations in the activin

receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. Nat Genet 13:189–195
Kjeldsen AD, Brusgaard K, Poulsen L, Kruse T, Rasmussen K, Green A, Vase P (2001) Mutations in the *ALK-1* gene and the phenotype of hereditary hemorrhagic telangiectasia in two large Danish families. Am J Med Genet 98:298–302
Lastella P, Sabba C, Lenato GM, Resta N, Lattanzi W, Gallitelli M, Cirulli A, Guanti G (2003) Endoglin gene mutations and polymorphisms in Italian patients with hereditary haemorrhagic telangiectasia. Clin Genet 63:536–540
Lesca G, Plauchu H, Coulet F, Lefebvre S, Plessis G, Odent S, Riviere S, Leheup B, Goizet C, Carette MF, Cordier JF, Pinson S, Soubrier F, Calender A, Giraud S (2004) Molecular screening of *ALK1*/*ACVRL1* and *ENG* genes in hereditary hemorrhagic telangiectasia in France. Hum Mutat 23:289–299
Lux A, Gallione CJ, Marchuk DA (2000) Expression analysis of endoglin missense and truncation mutations: insights into protein structure and disease mechanisms. Hum Mol Genet 9:745–755
McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J et al (1994) Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type1. Nat Genet 8:345–351
Olivieri C, Mira E, Delu G, Pagella F, Zambelli A, Malvezzi L, Buscarini E, Danesino C (2002) Identification of 13 new mutations in the *ACVRL1* gene in a group of 52 unselected Italian patients affected by hereditary haemorrhagic telangiectasia. J Med Genet 39:E39
Pece N, Vera S, Cymerman U, White RI Jr, Wrana JL, Letarte M (1997) Mutant endoglin in hereditary hemorrhagic telangiectasia type 1 is transiently expressed intracellularly and is not a dominant negative. J Clin Invest 100:2568–2579
Piantanida M, Buscarini E, Dellavecchia C, Minelli A, Rossi A, Buscarini L, Danesino C (1996) Hereditary haemorrhagic telangiectasia with extensive liver involvement is not caused by either HHT1 or HHT2. J Med Genet 33:441–443
Shovlin CL, Letarte M (1999) Hereditary haemorrhagic telangiectasia and pulmonary arteriovenous malformations: issues in clinical management and review of pathogenic mechanisms. Thorax 54:714–729
Shovlin CL, Guttmacher AE, Buscarini E, Faughnan ME, Hyland RH, Westermann CJ, Kjeldsen AD, Plauchu H (2000) Diagnostic criteria for hereditary hemorrhagic telangiectasia (Rendu-Osler-Weber syndrome). Am J Med Genet 91:66–67
Trembath RC, Thomson JR, Machado RD, Morgan NV, Atkinson C, Winship I, Simonneau G, Galie N, Loyd JE, Humbert M, Nichols WC, Morrell NW, Berg J, Manes A, McGaughran J, Pauciulo M, Wheeler L (2001) Clinical and molecular genetic features of pulmonary hypertension in patients with hereditary hemorrhagic telangiectasia. N Engl J Med 345:325–334
Van den Driesche S, Mummery CL, Westermann CJ (2003) Hereditary hemorrhagic telangiectasia: an update on transforming growth factor beta signaling in vasculogenesis and angiogenesis. Cardiovasc Res 58:20–31
Wallace GM, Shovlin CL (2000) A hereditary haemorrhagic telangiectasia family with pulmonary involvement is unlinked to the known HHT genes, *endoglin* and *ALK-1*. Thorax 55:685–690
Willemse RB, Mager JJ, Westermann CJ, Overtoom TT, Mauser H, Wolbers JG (2000) Bleeding risk of cerebrovascular malformations in hereditary hemorrhagic telangiectasia. J Neurosurg 92:779–784