

## Analysis of *ENG* and *ACVRL1* genes in 137 HHT Italian families identifies 76 different mutations (24 novel). Comparison with other European studies

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**Abstract** Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant disorder causing vascular dysplasias. About 70–80% of HHT patients carries mutations in *ENG* or *ACVRL1* genes, which code for a TGF $\beta$  receptor type III and I respectively. Molecular data on a large cohort of Italian HHT patients are presented, discussing the significance of missense and splice site mutations. Mutation analysis in *ENG* and *ACVRL1* genes was performed using single strand conformation polymorphisms (SSCP), denaturing high performance liquid chromatography (DHPLC) and subsequent direct sequencing. Overall, 101 mutations were found, with *ACVRL1* involved in 71% of cases. The highest number of mutations (28/101 subjects, 14/76 different mutations referring to both genes) was in *ACVRL1*,

exon 3. Mutation analysis was then extended to a total of 356 family members, and 162 proven to carry the mutation. New polymorphisms were identified in both genes, and evidence that *ENG* P131L change is not a disease-causing mutation was also provided. An *in silico* analysis was performed in order to characterize splice-site mutations. These results were compared to other European national studies and data from Italy, France and Spain were consistent for an higher incidence of *ACVRL1* mutations.

**Keywords** Hereditary hemorrhagic telangiectasia · HHT · *ACVRL1* mutation · *ENG* mutation

### Introduction

Hereditary hemorrhagic telangiectasia (HHT; Mutation Database: <http://137.195.14.43/cgi-bin/WebObjects/hht.woa/wa/default>) is an autosomal dominant disorder causing vascular dysplasias such as mucocutaneous telangiectases and arterovenous malformations (AVMs). Telangiectases may lead to epistaxes and gastrointestinal bleeding, which may be severe enough to require transfusions. Epistaxes and telangiectases are the most frequent symptoms, present in more than 95% of the patients. AVMs are mostly observed in liver (60%), lungs (18–70%) and brain (6%), and may cause severe life-threatening complications (Lesca et al. 2007). The phenotype is highly variable, even among members of the same family, and the disease displays age-related penetrance, with increased manifestations developing over a lifetime. About 70–80% of HHT patients carry mutations in either of two genes—*ENG* (OMIM #131195) (HHT1: OMIM 187300) or *ACVRL1* (OMIM #601284) (HHT2: OMIM 600376)—which code for a TGF $\beta$  receptor type III and I respectively, although David

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et al. 2007 recently demonstrated that BMP9 is the more effective ligand of ACVRL1. Evidence for a third and a fourth locus on chromosomes 5 and 7 respectively has also been reported (Cole et al. 2005; Bayrak-Toydemir et al. 2006a). Association of the HHT phenotype with juvenile polyposis (JPHT, #175050) and mutations in the *MADH4* gene (coding for SMAD4, involved in the TGF $\beta$  signaling pathway) have recently been demonstrated as well (Gallione et al. 2004). Moreover, *MADH4* mutations may be observed in HHT patients without a prior diagnosis of JP (Gallione et al. 2006).

Several papers have recently been published reporting the results of screening for mutations in *ENG* and *ACVRL1* genes, and more than 200 pathogenetic variations have been reported for each gene.

Many polymorphisms are also present in both genes (HHT Mutation database: [www.hhtmmutation.org](http://www.hhtmmutation.org)). A peculiar distribution of mutations has been reported: *ENG* mutations are more frequently found in patients from Northern Europe and the Americas, while Mediterranean populations have a majority of *ACVRL1* mutations (Olivieri et al. 2002; Brusgaard et al. 2004; Lesca et al. 2004, 2006; Abdalla et al. 2005; Kjeldsen et al. 2005; Letteboer et al. 2005, 2006; Bayrak-Toydemir et al. 2006b; Fernandez-L et al. 2006; Lenato et al. 2006; Bossler et al. 2006).

Here we report the results of our screening for mutations in *ENG* and *ACVRL1* genes in a group of 137 Italian HHT patients. Overall, we found a mutation in 101 subjects (76 different mutations), with *ACVRL1* involved in 71% of our cases. We also identified several new polymorphisms in both genes, and provide evidence that *ENG* P131L change is not a disease-causing mutation.

## Materials and methods

### Patients

We screened a total of 137 index cases; 123 with a known family history, and 14 sporadic cases (family SLL in which the *ENG* P131L was found is not included in the figures). For each family, we selected a single case in whom the diagnosis was “definite”, according to the diagnostic criteria suggested by the International HHT Advisory board, known as “Curaçao criteria” (Shovlin et al. 2000). After the identification of a mutation, all other available relatives were also studied.

In 117 of them the diagnosis was made by EB in Crema (CR) and by FP, LS, SC and CD in Pavia, and the clinical data were shared between both centres. In 20 cases, the diagnoses were made in other centres who were certainly informed about the diagnostic criteria. Six families were of

Croatian, Dutch, Hungarian, Turkish and Indian origin. Detailed data as to the geographical origin of the Italian families were also recorded.

### Molecular analysis

After informed consent (prior to inclusion in the study), DNA was extracted from 3 to 7 ml of peripheral blood from each subject, using standard protocols.

Exons (including intron-exon boundaries) 1–13 of *ENG* gene and 1–10 of *ACVRL1* gene were amplified by PCR using the primers reported in the GDB database (Human Genome Database. The Official World-Wide Database for the Annotation of the Human Genome. <http://www.gdb.org>) (*ENG* 1–12, including 9a and 9b) or published by Berg et al. 1997 (*ACVRL1* 2–10). Exon 13 of *ENG* was split into three different PCRs in order to completely cover this region. Primers for these PCRs were designed by C. O. using the software on the web PRIMER3INPUT (Access date is May 25th, 2007 for all these softwares, [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)) and are available on request, as are the amplification protocols for the 26 different PCRs.

Mutation screening was performed by SSCP (*ACVRL1* gene) and/or DHPLC (*ACVRL1* and *ENG* genes) analysis. At least two different SSCP were performed, using different polyacrilammide concentration (from 6 to 8%, acrilammide bisacrilammide ratio: 19:1; 29:1; 37,5:1) as suggested by Ravnic-Glavač et al (1994). The DHPLC was performed on a WAVE DNA fragment analysis system (Transgenomic, Cheshire, UK). The temperature of the oven for optimal heteroduplex separation at partial DNA sequence was obtained with the Wavemaker 4.0 software (Transgenomic). The DHPLC optimal temperatures are available on request.

Each time an abnormal pattern was found, direct sequencing was performed using the Big Dye terminator method combined with Taq FS (cycle sequencing reaction). Sequence was analysed on a ABI-PRISM 3700 DNA analyser (Applied Biosystems).

Each mutation was confirmed at least by a second sequence. When an unpublished missense mutation was found, we analysed 50 control DNAs and, when possible, we confirmed the cosegregation of the mutation with the disease in the family. We also checked if the amino acid was conserved in orthologous genes.

When a mutation was found in an “index case”, all other subjects belonging to his/her family were analysed using direct sequencing or endonuclease restriction digestion. The relatives (affected and non-affected) were all informed about the results, and genetic counselling was provided.

## Splice site scores

To evaluate if mutations found in the intron-exon boundary region affected the splice site, we performed four different analysis using software for splice site scores calculation present on the web:

SCORE 1: Splice site analyzer tool: <http://ast.bioinfo.tau.ac.il/SpliceSiteFrame.htm>

SCORE 2: Alex Dong Li's splice site score calculator (v 0.1): <http://www.genet.sickkids.on.ca/~ali/splicesitescore.html>

SCORE 3: Splice site score calculation: [http://rulai.cshl.edu/new\\_alt\\_exon\\_db2/HTML/score.html](http://rulai.cshl.edu/new_alt_exon_db2/HTML/score.html)

SCORE 4: MaxEntScan::score5ss for human 5' splice sites: [http://genes.mit.edu/burgelab/maxent/Xmaxentscan\\_scoreseq.html](http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html)

MaxEntScan::score3ss for human 3' splice sites: [http://genes.mit.edu/burgelab/maxent/Xmaxentscan\\_scoreseq\\_acc.html](http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq_acc.html)

All these tools are based on the paper by Shapiro and Senapathy (1987), Carmel et al. (2004) and Yeo and Burge (2004).

Access date is May 25th, 2007 for all these software programs.

## Results

Mutations and polymorphisms identified in our study are summarized in Tables 1 and 2 respectively. Nomenclature follows suggestions reported by den Dunnen and Antonarakis (2000). GenBank RefSeq-file accession number are NM\_000118.1 for *ENG* and NM\_000020.1 for *ACVRL1*. Nucleotide numbering uses the A of the ATG translation initiation start site as nucleotide +1.

In *ENG* we identified 26 different mutations in 29 subjects; 13 are, to the best of our knowledge, unpublished.

We found 12 nucleotide substitutions, 12 deletions and two insertions. As to their effects, 16/26 (61.5%) were truncating mutations, 5/26 (19.3%) were splice-site mutations, 4/26 (15.4%) were missense mutation and 1/26 (3.8%) was an in-frame deletion of 7 amino acids. Results of splice-site scores for these last mutations are reported in Table 3.

Mutations 6 and 8 were shared by three and two families respectively (see Table 1); only for the former could a common geographical origin be recorded.

We also identified nine different polymorphisms; two of them were, to the best of our knowledge, unpublished.

As to P131L, we identified a family in which two subjects were homozygous for this DNA change. The index case, a 9-year-old child, presented with epistaxes, which were present although limited to childhood also in the father and in two paternal uncles. Some cutaneous atypical

telangiectases were also present. This phenotype did not segregate with either homozygosity or heterozygosity for the P131L variant. No other *ENG* or *ACVRL1* mutation was found in this family, and it was therefore excluded from any calculation.

In *ACVRL1*, we identified 50 different mutations in 72 subjects; 11 are, to the best of our knowledge, unpublished.

The mutations can be classified as nucleotide substitution (36), deletions (8) and insertions/duplications (6).

As to their effect, 26/50 (52%) were amino-acid replacement, 20/50 (40%) were truncating mutations, 2/50 (4%) were deletions in frame of 2 amino acids and 2/50 (4%) affected a splice site (see Table 1). All the missense mutations involved conserved amino acids, when the sequence of orthologous genes were compared (*Rattus*, *Bos*, *Xenopus*, *Mouse*, and *Gallus*, data not shown). The results of splice site scores for the change-affecting splice sites are reported in Table 3.

Ten mutations (nos. 31,34,35,37,41,44,55,62,68,70 in Table 1) were found in more than one index case; all families carrying the same mutation were unrelated going back for at least four generations, but shared a common geographic origin, with the exception of mutation no. 44 (Table 1): ID1 is a sporadic case and ID287, ID701 and ID705 have a different geographical origin.

We identified six intronic polymorphisms (outside splicing regions); four of them are unpublished and were found in a small proportion of patients (<5%); we also determined the prevalence of g.IVS3 + 11 c > t polymorphisms (Olivieri et al. 2002) (see Table 2) which was in Hardy-Weinberg equilibrium.

In one of our patients, a history of colon cancer at age 34 suggested *MADH4* involvement; mutation analysis performed by C. Gallione identified a previously unreported *MADH4* mutation (R361L). The son of the index case carries the same mutation, shows a typical HHT clinical picture, but has no evidence of polyposis at age 19 (as assessed by colonoscopy). The healthy mother of the index case, as expected, does not carry the mutation; the father died because of a pancreatic cancer at age 56, and no biological samples were available for analysis.

We then compared our results to the screenings recently published by other groups on different European populations [Germany (Kuehl et al. 2005; Schulte et al. 2005; Wehner et al. 2006), Spain (Fernandez-L et al. 2006), Denmark (Brusgaard et al. 2004), France (Lesca et al. 2004, 2006), Netherlands (Letteboer et al. 2005)] and to the work of Lenato et al. 2006 on a smaller group of Italian patients (see Figs. 1,2).

The distribution of the mutations in the two genes [number of *ENG* mutations/total number of mutations] varied from 37% in Spain to 54% in the Netherlands. When considering the Italian population, *ENG* mutations were

**Table 1** Summary of *ENG* and *ACVRL1* mutation found in this study

Number	ID	Exon	Mutation	References
<b>ENG</b>				
1	423	2	c. 77del A (p. E26fsX42)	Unpublished
2	284	2	c. 96_102 del TCAGCCT (p. L32 fs: X40)	Unpublished
3	693	2	c. 97C > T (p.Q33X)	Lenato et al. 2006
4	359	2	c. 145G > T (p. V49F)	Lesca et al. 2004
5	36	2	c. 210 del G (L69fsX80)	Unpublished
6	56, 90, 718	3	c. 277C > T (p. R93X)	Cymerman et al. 2000 (+4)
7	662	3	c. 338 del G (p. L112 fs:X147)	Unpublished
8	135, 632	IVS3	g. IVS3 + 1 g > a (Splice Site) (p. Gly74_Tyr120del)	Pece et al. 1997 (+7)
9	368	IVS3	g. IVS3 + 3 del agtg	Unpublished
10	169 <sup>8</sup>	4	c. 495_496 del CC (p. P165 fsX332)	Unpublished
11	684	5	c. 526C > T (p. Q176X)	Unpublished
12	563	5	c. 574 del C (p. 191 fs:X221)	Unpublished
13	643	5	c. 590G > C (p. R197P)	Unpublished
14	140	6	c. 772 del T (p. Y258 fs: X359)	Fernandez-L et al. 2006
15	32	6	c. 774C > G (p. c258Y)	Unpublished
16	389	6	c. 780 Ins (Dup 768–775) (p. S260 fs X358)	Olivieri et al. 2006
17	377	IVS6	g. IVS 6 + 5 g- > c	Olivieri et al. 2006
18	52	7	c. 909_929 del (p. R304_I310 del in frame)	Lastella et al. 2003 (+1)
19	41	8	c. 1010C > G (p. S337X)	Unpublished
20	270	8	c. 1080_1083 del GACA (p. G360fs)	Gallione et al. 1998 (+3)
21	367	8	c. 1134G > A (p. A374A:splice site)	Letteboer et al. 2005 (+1)
22	326	IVS8	g. IVS8 + 1 g > a (p. G332fs)	Shovlin et al. 1997 (+1)
23	127	9a	c. 1144T > G (p. C382G)	Unpublished
24	245	11	c. 1478 del G (p. S492 fs X516)	Olivieri et al. 2006
25	198	11	c. 1550_1551 del TG (p. C516 fs X525)	Mc. Allister et al. 1995
26	138	11	c. 1684_1686 + 11 dup 13 bp (p. Q562 fs X570)	Unpublished
<b>ACVRL1</b>				
27	422 <sup>8</sup>	2	c. 50_53 del TGGT (p. L17X)	Unpublished
28	707	3	c. 143G > A (p. G48E)	Brusgaard et al. 2004
29	170	3	c. 144_145 ins G (p.G48 fs: X167)	Klaus et al. 1998 (+1)
30	258	3	c. 145 del G (p. G48 fs: X53)	Olivieri et al. 2002 (+1)
31	172, 241	3	c. 152G > A (p. C51Y)	Klaus et al. 1998 (+2)
32	131	3	c. 164_169 del TGGTGC (del L55V56 in frame)	Olivieri et al. 2006
33	105	3	c. 172G > T (p.E58X)	Olivieri et al. 2002 (+1)
34	199, 476	3	c. 199C > T (p. R67W)	Olivieri et al. 2002 (+3)
35	263, 380, 435	3	c. 200G > A (p. R67Q)	Berg et al. 1997(+5)
36	280	3	c. 203 ins G (p.G68 fs:X166)	Olivieri et al. 2006
37	407, 598	3	c. 205_209 dup TGCGG (p. G70 fs:X123)	Unpublished
38	633	3	c. 230G > A (p. C77Y)	Unpublished
39	603	3	c. 235G > A (p. G79R)	Olivieri et al. 2006
40	704	3	c. 266G > A (p. C89Y)	Unpublished
41	50, 255, 290 310, 464, 640 686, 813, 818, 827	3	c. 289_294 del CACAAC (p. H97-N98 del in frame)	Olivieri et al 2002
42	38	IVS 3	g. IVS3 -3 (c. 314 -3) C > G (Splice site)	Prigoda et al. 2006
43	64	4	c. 321 del A (p. Q107 fs X121)	Olivieri et al. 2006
44	1, 287, 701, 705	4	c. 430C > T (p. R144X)	Paquet at al. 2001 (+7)

**Table 1** continued

Number	ID	Exon	Mutation	References	
45		720	4	c. 435 del G (p. R145fs: X164)	Fernandez-L et al. 2006
46		249	4	c. 448C > T (p. Q150X)	Lenato et al. 2006
47		412	4	c. 476_477 del AG (p. G158 fs: X167)	Unpublished
48		711	IVS 4	g. IVS4 +1 G > A (Splice site)	Unpublished
49		11	5	c. 617A > G (p. E206G)	Unpublished
50		247	6	c. 650G > A (p. W217X)	Bossler et al. 2006
51		282	7	c. 810_822del CACGCAGCTGTGG (p. S270fs:X297)	Olivieri et al. 2002
52		362	7	c. 824_827 dup GGCT (p. L273fsX392)	Olivieri et al. 2006
53		716	7	c. 853 dup C (p. S284fs: X107)	Unpublished
54		242	7	c. 858C > A (p. Y286X)	Olivieri et al. 2002 (+3)
55	594, 626	7	7	c. 924C > A (p. C308X)	Berg et al. 1997(+2)
56		297	7	c. 988G > A (p. D330N)	Letteboer et al. 2005
57		187	7	c. 988G > T (p. D330Y)	Olivieri et al. 2002 (+2)
58		266	7	c. 1031G > T (p. C344F)	Abdalla et al. 2000 (+1)
59		634	7	c. 1048G > A (p. G350S)	Schulte et al. 2005
60		148	8	c. 1054G > C (p. A352P)	Olivieri et al. 2002
61		300	8	c. 1080_1098 dup 19bp (p. D360 fs X397)	Olivieri et al. 2002
62	20, 621, 667	8	8	c. 1120C > T (p. R374W)	Berg et al. 1997 (+18)
63		722	8	c. 1121G > A (p.R374Q)	Abdalla et al. 2003 (+4)
64		639	8	c. 1126A > G (p. M376V)	Lesca et al. 2004 (+1)
65		388	8	c. 1127T > A (p. M376K)	Olivieri et al. 2006
66		129	8	c. 1133C > T (p. P378L)	Olivieri et al. 2002
67		593	8	c. 1139T > G (p. V380G)	Letteboer et al. 2005
68	209, 588	8	8	c. 1199C > A (p.A400D)	Olivieri et al. 2002
69		40	8	c. 1221G > T (p. E407D)	Abdalla et al. 2000 (+2)
70	552, 687	8	8	c. 1232G > A (p. R411Q)	Johnson et al. 1996 (+11)
71		608	10	c. 1385C > G (p. S462X)	Abdalla et al. 2004
72		313	10	c. 1435C > T (p.R479X)	Lesca et al. 2004 (+5)
73		438	10	c. 1438C > T (p. L480F)	Unpublished
74		583	10	c. 1445C > A (p. A482E)	Unpublished
75		168	10	c. 1450C > T (p. R484W)	Trembath et al. 2001 (+3)
76		251	10	c. 1451G > A (p.R484Q)	Unpublished

Numbers after references indicate how many additional times the mutation has been published

§ the non-Italian patients

found in 29 % in our cohort and in 40% in the study by Lenato et al. 2006.

*ENG* mutations are almost evenly distributed in the various exons coding for the extracellular domain, while the *ACVRL1* gene shows clustering of mutations in exons 3, 7, 8, and this uneven distribution is confirmed in all the European samples studied. Conversely, exon 5 is consistently less involved in all published reports.

## Discussion

The analysis of a group of 137 index patients affected with HHT allowed us to identify 76 different mutations in 101

subjects, 24 unpublished; 162 out of the 356 family members subsequently tested were proven to be mutation carriers.

All but two of the cases (one Indian, one Swedish) were of Italian ancestry (see Table 1). On the basis of these data and of other recent reports, the number of different known mutations arises to over 200 for each gene; a further increase in the number of new mutations in both *ENG* or *ACVRL1* is to be expected.

A “founder effect” for specific mutations observed with high frequency was suggested in the Netherlands Antilles (*ENG*: c.1238G > T; g.IVS1 + 1 g > a; Gallione et al. 2000), France (*ACVRL1*: c.1112\_1113 dupG; Lesca et al.

**Table 2** Summary of *ENG* and *ACVRL1* polymorphisms found in this study

Exon	Position	Effect	Genotypes	References	%
<b>ENG</b>					
2	c. 207 G > A	p. L69L	3 Het	Shovlin et al. 1997 (+3)	11
IVS2	g.IVS2 + 22c > t	No	1 Het	Bossler et al. 2006	
IVS2	g.IVS2 + 25g > t	No	2 Het	Prigoda et al. 2006	5
4	c. 392 C > T	p. P131L	2 Hom	Cymerman et al. 2003 (+3)	
5	c. 572 G > A	p. G191D	8 Het	Lesca et al. 2004 (+2)	6,4
IVS7	g.IVS7 + 25_26 ins cctccc	No	1/10 Het	Lesca et al. 2004 (+1)	42,1
8	c. 1023 C > A	p. I341I	1 Het	Novel	
8	c.1029C > T	p. T343T	9 Het	Shovlin et al. 1997 (+2)	5
8	c. 1060C > T	p. L354L	1 Het	Prigoda et al. 2006	1,6
<b>ACVRL1</b>					
IVS1	g.IVS1-23 c > t	NO	6/16 Het	Novel	
2	C(ATG -38)T	NO	6 Het	Abdalla et al. 2003 (+1)	14
IVS3	g. IVS3 + 11 c > t	NO	50% c/t, 18%t/t	Olivieri et al. 2002 (+1)	
IVS4	g. IVS4-22 a > g	NO	1 Het	Novel	
IVS5	g. IVS5 -58: ins t	NO	3 Het	Novel	
IVS5	g. IVS5 -53 c > t	NO	2 Het	Prigoda et al. 2006	

References and figures in brackets are as in Table 1

% as reported by references, *Het* Heterozygous, *Hom* Homozygous

2004) and Denmark (*ENG*: c.360C > A; Brusgaard et al. 2004).

We observed a small cluster in the North Italian county of Bergamo (*ACVRL1*: c.289 del CACAAC, p. H97-N98 del in frame; Table 1, mutation 41) with the same mutation in ten apparently unrelated families with 40 affected subjects, from a population for the county of 1,021,700 in 2004. Other HHT families with different mutations are known in the same area.

This cluster may partially explain the higher percentage of *ACVRL1* mutation in our cohort as compared to the results of Lenato et al. 2006.

We also identified several polymorphisms in both genes by DHPLC analysis, a method that does not allow homozygote identifications; as most of them are intronic variations, outside splicing sites, and thus are unlikely to have any effect on the protein, we did not check if these polymorphisms were in Hardy-Weinberg equilibrium.

The Endoglin P131L substitution was reported as a HHT-causing mutation (Kjeldsen et al. 2005; Fernandez-L et al. 2006; Cymerman et al. 2003) and was subsequently listed as a variant (Abdalla and Letarte 2006). Homozygous Endoglin null mice have an embryonic lethal phenotype (Arthur et al. 2000), and a previous report showed no live children homozygous for *ENG* mutation in a consanguineous family where both parents were affected (Karabegovic et al. 2004). Our results (no segregation with phenotype, presence of two homozygotes) provide conclusive evidence that P131L is probably a poly-

morphism whose biological effect, if any, is still to be assessed.

The *ENG* protein contains a large extracellular domain (encoded by exons 1–12), a transmembrane and an intracellular domain (exons 13 and 14). The mutations are distributed almost evenly in all exons with the exception of a clear lower involvement of exons 1 and 12 to 14 (HHT mutation database; Wehner et al. 2006); our data support this distribution (Fig 1).

Most mutations result in a truncated protein (61.5% in our sample), which is in agreement with the model of haploinsufficiency proposed for the pathogenesis of the disease (Abdalla and Letarte 2006). This model is supported by studies in KO mouse (Arthur et al. 2000; Bordeau et al. 2000) and by the demonstration of a 50% level of protein in HUVEC HHT1 cells (Pece et al. 1997; Cymerman et al. 2000).

A number of DNA changes resulting in amino acid variation but interpreted as polymorphisms have also been reported as [*MTHFR*, A222V]. A high population frequency of the observed change “per se” cannot be held as a demonstration that the variation has no effect on the protein function and in turn on the general phenotype of the subject carrying it (Paquet et al. 2001). For instance, the [*MTHFR*, A222V] will result in a protein with definitely modified behaviour, whose presence is associated with vascular disorders and other diseases.

Endoglin polymorphisms resulting in amino acid changes and not causing HHT (as a second obviously pathogenetic

**Table 3** Splice scores for splice site mutation

Gene	Exon	Mut	Sequence	Type	Score1			Score2	Score 3	Score4			
					S	BP	ΔG			Maxent	MDD	MM	WMM
ENG	IVS3	WT	TACgtgagt	5'	81.03	8	-4.2	83.2	7.2	7.77	12.28	6.13	6.80
		8	TACatgagt		63.19	7	-1.8	65.0	-3.5	-0.41	4.10	-2.05	-1.38
		9	TACatgtgt		70.63	8	-0.6	71.9	4.6	1.40	6.98	1.55	3.86
	IVS6	WT	TGGgtgagt	5'	85.28	8	-9.3	88.9	8.8	8.73	13.18	8.36	7.38
		17	TGGgtgact		72.27	7	-5.6	74.6	5.1	0.99	3.68	3.49	3.54
	IVS8	WT	GCGgtaagg	5'	85.01	6	-7.4	87.2	8.0	9.63	13.08	9.59	8.59
21		GCAgtaagg	72.59		5	-4	74.8	5.1	4.69	10.38	5.58	5.42	
22		GCGataagg	67.17		5	-1.7	69.0	-2.7	1.45	4.90	1.41	0.41	
ACVRL1	IVS3	WT	tcagtgtcccctccctcagCCA	3'	74.84	-	-	84.1	9.1	6.88	/	7.69	9.30
		42	tcagtgtcccctccctcagCCA		64.26	-	-	72.4	-1.3	-4.69	/	-0.59	0.39
	IVS4	WT	GGGgtatgg	5'	74.66	7	-4.7	76.3	5.4	5.84	7.88	4.93	5.71
		48	GGGatatgg		56.82	6	-1.2	58	-5.3	-2.34	-0.30	-3.26	-2.47

SCORE 1: S Splice-site score. Best possible score = 100

BP number of H-bonds between U1 and the 5' splice site

ΔG free energy. Best score of 5' splice site = -9.6

SCORE 2: A 100% match to the mammalian 3' and 5' splice site would have a score of 100

SCORE 3: A 100% match to the mammalian 3' splice site would have a score of 14.2. The mean score of the 3' splice site in constitutive exons is 7.9

SCORE 4: Maxent Maximum entropy model score

MDD Maximum dependence decomposition model score

MM First-order Markov model score

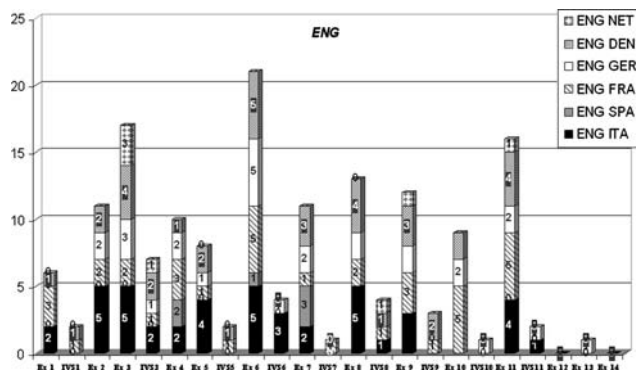
WMM Weight matrix model score

A perfect 5' splice site would have a score of 12.6. The mean score of the 5' splice site in constitutive exons 8.1

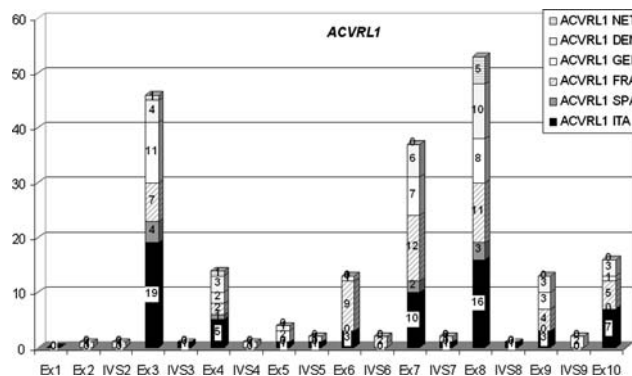
The ideal MaxEnt score is 11.81 for a 5' splice site and 13.59 for a 3' splice site. For the other models, the higher is the score, the higher is the probability that the sequence is a true splice site

WT wild type sequence

Mut mutation numbers (as in Table 1)



**Fig. 1** Distribution of *ENG* mutation in six European countries. Figures refer to the number of different mutations found. *NET* Netherlands, *DEN* Denmark, *GER* Germany, *FRA* France, *SPA* Spain, *ITA* Italy. For references, see the text



**Fig. 2** Distribution of *ACVRL1* mutation in six European countries. Figures refer to the number of the different mutations found. *NET* Netherlands, *DEN* Denmark, *GER* Germany, *FRA* France, *SPA* Spain, *ITA* Italy. For references, see the text

mutation in the same gene or in *ACVRL1* gene is concurrently present) should thus be reported and studied to demonstrate if they have any effect on protein function. This situation is

observed in cases ID170, ID131 and ID300, in which a *ACVRL1* mutation was associated to the *ENG* G191D polymorphism. Until this datum is obtained, we cannot

formally exclude the possibility that (minor) changes in protein activity may exert an addictive effect on the HHT phenotype or may be a risk factor for different phenotypes.

The ALK1 protein consists of a small extracellular domain (encoded by exons 2,3, partially 4), a short transmembrane domain (exons 4,5 partially), and a large intracellular domain including a glycine-serine rich (GS) domain (exon 5) and the serine/threonine kinase (S/T-K) domain (exons 5 to 10).

The mutations are unevenly distributed, with exons 3,7,8 carrying a larger number of mutations (61.46% of the total number of *ACVRL1* mutations, as calculated from the HHT mutation database). This trend is observed in all European countries, and also in our cohort of cases, with the only peculiarity of a higher number of mutations in exon 3.

In fact in our group of HHT2 patients, we found 28/72 subjects (39%) to be carriers of exon 3 mutations; if only the different *ACVRL1* mutations are considered, exon 3 still contains 14/50 mutations (28%), versus 22% in Europe and in the database.

In exon 5, only 5 mutations are known; among them, the only mutation identified in the GS domain (Harrison et al. 2003) was found in a patient with PAH but, apparently, without clinical signs or family history of HHT. Other mutations in exon 5 generate a truncated protein or are in the S/T-K domain, as our mutation 49 in Table 1.

For two mutations in the S/T-K domain (S333I and C344Y), Gu et al. (2006) suggested a dominant negative effect, on the basis of the evaluation of ALK1 expression and activity in monkey and human cell lines. We found a C344F mutation (Table 1, n.58) in which cysteine is replaced by phenylalanine instead of tyrosine as for the study by Gu et al.; the two substituted amino acids are structurally similar, and the hypothesis of a dominant negative may thus be considered for our mutation too. Moreover, the same authors demonstrated that the R411Q mutation results in a protein which retains its activity, although at a reduced level.

Very interestingly, Fernandez-L et al. (2005) demonstrated on BOECs (Blood Outgrowth Endothelial Cells) from HHT2 patients carrying the R374W missense mutation an ALK1 amount similar to that of controls by western blot analysis; this result suggests that some missense mutations may be expressed. Both the R374W and the R411Q are present in our cohort (Table 1, mutation 62 and 70).

The use of the SIFT Tool allowed Prigoda et al. (2006) to show that the vast majority of, but not all, ALK1 missense mutations lead to altered structures, which might imply loss of function.

Altogether, these data indicate that the pathogenesis of HHT2 may be more complex than previously thought; haploinsufficiency may not be the only disease-causing mechanism, and different mutations may lead to (slightly) different phenotypes.

In vivo studies and the development of functional biological tests will settle this point.

In the *ACVRL1* gene we did not find any polymorphism resulting in amino acid change, in agreement with previously published data.

We found seven different DNA changes (two in *ACVRL1*) occurring at a splice site; as all of our work was performed on DNA samples, and data on the expression were not available, we studied these changes in silico using four different tools (Table 3). They were considered as disease-causing mutations when segregating with the disease in the family, and when the power of the splicing was reduced to an extent similar to the result reported by Roca et al. 2003.

A mutation in either *ENG* or *ACVRL1* was found in 75.6% of the Italian index cases and in 2/6 of the non-Italian patients; in addition, 1 *MADH4* mutation was found in a family selected because of the phenotype of the index case. The reasons for failure in identification of a mutation in a proportion of the remainder of cases have been extensively and clearly discussed by Lesca et al. (2004) and include technical limits and genetic heterogeneity.

The comparison of our data with those of other European countries indicates that Italy, Spain and France homogeneously show an higher percentage of *ACVRL1* mutations with a narrow range: 60–62%. In other countries such as Germany, Denmark and The Netherlands, the percentage of HHT2 patients is lower, and the picture seems less homogeneous, as the percentage of *ACVRL1* mutations ranges from 45 to 57%. Knowledge of the relative distribution of the mutations in the two genes is helpful in developing strategies for testing HHT patients.

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