

ONLINE MUTATION REPORT

Identification of 13 new mutations in the *ACVRL1* gene in a group of 52 unselected Italian patients affected by hereditary haemorrhagic telangiectasia

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Hereditary haemorrhagic telangiectasia (HHT) (OMIM 187300) is an autosomal dominant disorder caused by mutations in either of two genes, endoglin (*ENG*, OMIM 131195) (HHT1) and activin A receptor type II-like 1 (*ACVRL1*, OMIM 601284) (HHT2). Evidence for a third locus has also been reported.¹

The product of the *ACVRL1* gene is a type I receptor for the TGF-beta group of ligands; it is associated with the TGF-beta or activin type II receptors and the complex binds TGF-beta or activin. It is highly expressed in endothelial cells, lung, and placenta, as endoglin, mutations of which are observed in HHT1; endoglin is supposed to sequester TGF-beta and present the ligand to activin A receptor type II-like 1 plus a type II receptor.² Mutations in *ENG* and *ACVRL1* may cause HHT1 or HHT2, respectively, by disrupting this complex.

The clinical presentation, indistinguishable between HHT1 and HHT2, typically includes epistaxis and telangiectasia, and the diagnosis can be considered to be confirmed, according to the proposal of Shovlin *et al*,³ if three of the four suggested diagnostic criteria (epistaxis, telangiectasia, visceral lesions, positive family history) are present. The phenotype is highly variable and penetrance is complete by the age of 40 years.³

Arteriovenous (AV) fistulae are frequently observed in the liver (8% of patients),³ lungs (20%),⁶ and brain⁵ and may cause severe life threatening complications. Neurological complications (strokes, cerebral abscesses, seizures) may be prevented with appropriate treatment of the pulmonary arteriovenous malformations (PAVMs). A higher risk for lung involvement has been suggested in patients carrying mutations in the *ENG* gene,⁷ while in some families with a peculiar liver involvement, mutations in *ACVRL1* have been described.⁸ The involvement of the latter gene has also been reported in a single patient with a pituitary tumour⁹ without any overt sign of HHT and in families with HHT and pulmonary hypertension.¹⁰

The number of mutations identified so far is limited; in particular for *ACVRL1* only 29 mutations have been reported (table 1), always in single patients or in very small series. The mutations are scattered along the gene; there is not, at present, any mutation occurring with significantly higher frequency, and exons 3, 7, and 8 seem to harbour about 59% of the known mutations.²⁻⁸⁻¹⁵ Based on these data, we decided to test our group of patients for the presence of mutations in these three exons, to verify if analysis of part of the *ACVRL1* gene may result in the attribution of a consistent number of unselected patients to HHT2.

We describe here SSCP and/or dHPLC screening for exons 3, 7, and 8 in a group of 52 unselected HHT patients.

MATERIALS AND METHODS

Patients affected by HHT were diagnosed by EB or EM using the published criteria.³ All patients are of Italian ancestry and details on the city of origin of each family were also obtained. Detailed clinical data will be reported elsewhere.

We collected blood samples, after informed consent, from 54 index cases and from available relatives at risk of being affected on the basis of the family pedigree; two of them belonged to large families in which linkage analysis showed the involvement of *ENG* and were excluded from *ACVRL1* analysis.

DNA from the remaining 52 index cases was extracted by routine techniques; exons 3, 7, and 8 were amplified with the primers reported by Berg *et al*¹¹ and analysed by SSCP with two different running conditions¹⁶ in order to reduce false negative results (exon 3, which contains a common polymorphism) or dHPLC plus SSCP (exons 7 and 8).

In each experiment, a control for which the presence of a wild type sequence had previously been shown by direct sequencing and, for exon 3, a carrier of the common polymorphism were also included.

All subjects displaying an SSCP (or dHPLC) pattern different from controls or carriers of the common polymorphism of exon 3 were sequenced, using the Big Dye terminator method associated with *Taq* FS enzyme (Cycle Sequencing reaction) and an ABI-PRISM 3700 DNA analyser (Applied Biosystems).

The sequences obtained were compared to the reported gene sequence (working draft NT 009609) using the BLASTN program.

RESULTS AND DISCUSSION

The 13 different mutations in exons 3, 7, and 8 found in 16 unrelated probands after screening the 52 HHT patients are reported in table 2.

Only the mutation found in case LPV has been already reported,¹¹ while the others are all new. Four mutations (cases LL, PS, SA, VC, table 2) cause a frameshift and introduce a stop codon and two mutations (cases Dit201 and MoA, table 2) introduce a stop codon; thus these six mutations are certainly responsible for the production of a truncated and defective protein.

Two mutations occurred twice (delH97N98 and R67W), but no relationship could be found up to the great grandparents for each couple of families; however, in both cases the family names originate from closely related geographical areas in north Italy.

Del H97N98 deletes two amino acids of the extracellular domain, where N98 is a potential glycosylation site; the two amino acids are conserved in *Mus musculus* and *Rattus norvegicus* and N98 is also conserved in *Xenopus laevis* and *Gallus gallus*. R67 is conserved in mouse and rat while a homologous amino acid is present in *Xenopus laevis* and *Gallus gallus*. The presence

Abbreviations: HHT, hereditary haemorrhagic telangiectasia; AV, arteriovenous; PAVM, pulmonary arteriovenous malformation, STR, short tandem repeat

Table 1 Summary of the mutations already published for the *ACVRL1* gene

Exon	Mutation	Effect	References
2	37 delC	L13fs (+1aa) and stop	10
3	143G>A/145del G/147insT	G48E; A49P	13
3	140 ins G	fs and premature stop	12
3	150G>T	W50C	11
3	152G>A	C51Y	12
3	200G>A	R67Q	11
3	231C>G	C77W	12
3	286A>G	N96D	12
4	400delG	fs and premature stop in exon 4	12
4	406-9 delGGTG	fs and premature stop in exon 4	12
4	423G>A	W140X	11
4	475G>T	E159X	11
6	694-6 delTCC	del S232	2
6	696-698del CTC	del S232	15
6	759-761delCGA	del D254	10
7	865 insT	fs and stop in exon 7	11
7	998G>T	S333I	11
7	1031G>A	C344Y	13
7	924C>A	C308X	11
8	1120C>T	R374W	11
8	1126T>G	M376R	2
8	1193T>A	I398N	14
8	1221G>T	E407D	13
8	1231C>T	R411W	10
8	1232G>A	R411Q	2
9	1207C>A	P424T	11
10	1445C>T	A482V	9
10	1450C>T	R484W	10
10	1468C>T	Q490X	10

fs: frameshift

of the same change at a conserved amino acid in two unrelated (or distantly related) families is a point in favour of its causal relation to the disease. The same changes were not observed in any other allele examined (n=50).

P301, carrying the R67W mutation (table 2) was found in a family previously reported as unlinked to either chromosome 9 or 12.¹⁷ After re-evaluation of all family members according to the updated diagnostic criteria³ and a new linkage analysis, the evidence for exclusion of chromosome 9 remained strong, while the evidence for the exclusion of chromosome 12 was not significant; thus, the proband was included in the study. The extensive liver involvement in this family is confirmed and it is noteworthy that intrahepatic AV shunts are also

present in the apparently unrelated family sharing the same mutation (case MC, table 2), which comes from the same geographical region. A different missense mutation at the same position, R67Q, was reported by Berg *et al.*,⁷ confirming that the substitution of this arginine causes HHT2. In addition, Lux *et al.*¹⁸ have shown the loss of signalling activity in vitro for mutations C51Y (case 3 in table 2) and R67Q.

The amino acid changes D330Y, C344F, A352P, P378L, and A400D (table 2, fig 1) all occur at highly conserved residues (*Rattus norvegicus*, *Xenopus laevis*, *Gallus gallus*, *Drosophila melanogaster*), so they are very likely to produce protein changes causing the disease. None of them has been found in any other examined allele (n=50).

Table 2 Summary of the mutations found

Nº	ID	Sex	Mutation	Phenotype
<i>Exon 3</i>				
1	LL	M	145 ins G	Frameshift+Stop: X167
2	PS	M	145 del G	Frameshift+Stop: X53
3	LPV	M	152G>A	C51Y
4	Dii201	M	172G>T	E58X
5	MC	F	199C>T	R67W
6	P301	F	199C>T	R67W
7	PP	M	289-294del CACAAC	Del H97N98 in frame
8	VG	F	289-294del CACAAC	Del H97N98 in frame
<i>Exon 7</i>				
9	SA	F	809-821delCACGCAGCTGTGG	Frameshift+Stop: X297
10	MoA	F	858C>A	Y286X
11	LG	F	988G>T	D330Y
12	SL	M	1006G>T	C344F
<i>Exon 8</i>				
13	IAC302	M	1054G>C	A352P
14	VC	F	1080-99dupl GCACTCACAGGGCAGCGAT	Frameshift+Stop: X396
15	FP	M	1133C>T	P378L
16	M203	M	1199C>A	A400D

Ep = epistaxis; Te = telangiectasia; L = intrahepatic arteriovenous shunts.

(1) 328	HRDFKSRNVLVKSNLQCCIADLGLAVMHSQ	357
(2) 329	HRDLKSRNVLVKSNLQCCIADLGLAVMHSQ	358
(3) 331	HRDLKSNLVLVRLKNGQCCIADLGLAVMHSQ	360
(4) 329	HRDLKSNLVLVRLKNGQCCIADLGLAVMHSQ	358
(5) 391	HRDLKSNLVLVTSNGSCVIADFGGLAVTHSH	420
(1) 375	YMAPEVLDEQIRTD [*] CFESYKWTDIWAFGLV	404
(2) 376	YMAPEVLDEQIRTD [*] CFESYKWTDIWAFGLV	405
(3) 378	YMAPEVLDETIQVDC [*] FDSYKRVDIYAFGLV	407
(4) 376	YMAPEVLDETIQAD [*] CFDSYKRVDIWAFGLV	405
(5) 438	YMAPEVLDESIDLECFEALRR [*] TDIYAFGLV	467

Figure 1 Alignment of amino acid sequences for genes orthologous to *ACVRL1*. (1) *Homo sapiens*. (2) *Rattus norvegicus*. (3) *Xenopus laevis*. (4) *Gallus gallus*. (5) *Drosophila melanogaster*. Amino acids identical to the human sequence are in bold. Conserved substitutions are underlined. * indicates the position of the missense mutations of cases 11, 12, 13, 15, and 16.

The cosegregation of the mutations with the disease was analysed and shown in families of cases LPV, Dit201, MC, P301, VG, and Iac302, for which other affected members were available.

The genotypes of the common polymorphism of exon 3 c(intron 3 5'IVS+11)t (cp3), reported in the HHT mutation database, but not formally published, were found equally distributed in the whole group of HHT patients and in the control population (CC/CT/TT: 33.9/49.0/17.1 in patients and 30.8/53.8/15.4 in controls); the overall C allele frequency is 0.58 and Hardy Weinberg equilibrium is conserved. In the families of cases Dit201, P301, and MC, cp3 did not segregate with the disease.

We also analysed the short tandem repeat (STR) D12S1677 which is located between exons 9 and 10; the size of the alleles was randomly distributed among the 52 probands and the probands of the two families carrying the same mutations shared one (cases PP/VG in table 2) or both D12S1677 alleles (cases MC/P301 in table 2).

The 29 mutations in the *ACVRL1* gene reported so far in patients affected by HHT2 (table 1) are distributed in eight of the nine coding exons, but exons 3, 7, and 8 contain 59% of them. No mutations have been reported more than twice and no data on geographical distribution are available. Owing to the small number of mutations identified and to the extremely wide phenotypic variability, no detailed phenotype-genotype correlation is known.

A much lower incidence of lung AV malformation in *ACVRL1* linked families was suggested by Berg *et al.*⁷ but recently Kjeldsen *et al.*⁸ reported a T1193A (exon 8) mutation in a family with a high prevalence of PAVM and Trembath *et al.*¹⁰ identified *ACVRL1* mutations in HHT patients with clinical manifestations typical of primary pulmonary hypertension.

McDonald *et al.*⁸ reported in a single large family that the mutation G998T (exon 7) was associated with a significantly higher liver involvement, with 17% of screened patients showing liver AV fistulas. They proposed that these clinical findings may be specific for the mutation, and stressed the extreme variability present even in subjects carrying the same mutation.

Intrahepatic AV shunts were present in six of the 10 patients carrying mutations in the *ACVRL1* gene who agreed to undergo liver ultrasound examination. A positive family history for similar liver involvement is present in the families of cases P301, MC, and FP. In the same set of patients, lung x rays and pulse oximetry consistently yielded normal results. Our findings suggest the hypothesis that mutations in the *ACVRL1* gene may be associated with higher risk of liver AV malformations.

Key points

- Our work aimed to identify new mutations in the *ACVRL1* gene, and to collect data on liver involvement in HHT patients carrying mutations in this gene.
- We studied exons 3, 7, and 8 of *ACVRL1* by SSCP and/or dHPLC in a group of 52 unselected HHT patients diagnosed according to recently published criteria. All patients are of Italian background. Samples showing abnormal patterns with either method were sequenced.
- We observed 13 new unpublished mutations (two of them occurring twice); a previously reported mutation was also observed. The 16 mutations were distributed as follows: eight mutations in exon 3 and four mutations in both exons 7 and 8. Among the 16 patients thus identified as HHT2, 10 agreed to undergo liver ultrasound examination and six were found to be carriers of intrahepatic arteriovenous shunts.
- A review of published reports showed that 17/29 reported mutations were localised in exons 3, 7, and 8 of the *ACVRL1* gene in HHT2 patients; our results confirm that these exons carry a large number of mutations and that the mutations related to HHT2 are mostly different from each other. The presence of intrahepatic arteriovenous shunts was also found in a significant number of patients carrying the mutations.

Exon 3 contains information for the extracellular domain, while exons 7 and 8 contain information for a portion of the intracellular tyrosine kinase domain; however, at present, it is not possible to correlate precisely changes in the different segments of the protein with specific clinical symptoms.

Testing by SSCP and/or dHPLC exons 3, 7, and 8 of *ACVRL1* and sequencing only cases showing abnormal patterns allowed us to show that 30.7% of a group of 52 unselected HHT Italian patients may be classified as HHT2.

The prevalence of the disorder in the population is not well determined, probably because of the wide phenotypic variability; the best available estimates are those for a well defined region of Haut-Jura, Lyon, France⁵ and for the county of Fyn, Denmark,¹⁴ in which a prevalence of 15.6 per 100 000 was calculated. In our sample, the geographical origin of the families was evenly distributed over the whole of Italy so there is no evidence for a specific geographical aggregation of the disease in Italy as in France⁵; preliminary unpublished data by EB indicate a prevalence of 18/100 000 in the region of Piacenza, in northern Italy.

The probands from two pairs of families (MC and P301, and PP and VG, respectively, table 2) shared the same mutations, one or two alleles at the intronic D12S167 polymorphism and at the common polymorphism, and came from the same geographical area. Thus, they are very like to have a common ancestor.

In conclusion, we report a consistent number of new mutations in the *ACVRL1* gene identified in a large series of unselected Italian HHT patients; for six mutations an association with intrahepatic AV shunts was also documented. Our results contribute to the understanding of the spectrum of *ACVRL1* mutations leading to HHT2 and the high rate of intrahepatic AV shunts in HHT2, if confirmed, will have relevant implications for genetic counselling and clinical management of patients.

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The last two authors contributed equally to this work. We wish to thank the patients and their families for their cooperation and the association "Fondazione Italiana HHT-Onlus Onilde Carini" for their

support. We thank Mr A Pilotto for his skilful collaboration in performing dHPLC. This work was partially supported by "IRCCS Policlinico S Matteo-Pavia, Italy, grant "ricerca corrente" to EM. Data access: HGMD, Human Gene Mutation database, (<http://archive.uwcm.ac.uk>); BLAST, (<http://www.ncbi.nlm.nih.gov/BLAST>); NCBI codes for protein alignment (fig 1): (<http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=Protein>). *Homo sapiens*: 4557243 (NP_000011), *Rattus norvegicus*: 11967973 (NP_071886), *Xenopus laevis*: 2439947, (AAB71328), *Gallus gallus*: 14270376 (CAC39433); *Drosophila melanogaster*: 642862 (AAA61946).

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PostScript

BOOK REVIEW



Molecular Cytogenetics Protocols and Applications

Methods in Molecular Biology Volume 204. Editor Yao-Shan Fan. (Pp 424; \$135.00.) Totowa, NJ: Humana Press. 2002. ISBN 1-58829-006-9.

This book is a very up to date manual covering the background, methodologies, and applications of molecular cytogenetics techniques. The emphasis is on the diagnostic applications of FISH in the many areas of medicine on which it impinges, including paediatrics, fetal and reproductive medicine, pathology, haematology, oncology, and, of course, medical genetics. With 27 chapters and over 60 authors, all of whom are experts in their field, this book clearly shows how far molecular cytogenetics techniques have developed over the last two decades.

The book is divided into three parts. Part 1 covers the basic concepts and techniques. The opening chapter by the editor Yao-Shan Fan provides a very helpful overview of the scope of the book and an extensive set of references for further reading. The following chapters cover probe labelling (DNA and RNA probes) and basic FISH techniques. The second part of the book is devoted to evolving techniques and applications and includes chapters on microdissection, PRINS, SKY FISH, M FISH, CGH, colour banding FISH, fibre FISH, multi-telomere FISH, fluorescence genotyping for telomeric regions, and microarray CGH. Special applications of molecular cytogenetic techniques in chromosomal disorders are covered in part 3 of the book. These include chapters on the application of FISH to the

delineation of marker chromosomes and the diagnosis of microdeletion syndromes. Other chapters cover FISH interphase nuclei screening for prenatal diagnosis including preimplantation diagnosis and fetal cells in the maternal circulation, in addition to the interphase FISH screening of routine amniotic fluid samples. This section concludes with a chapter on the application of FISH and CGH in reproductive pathology. The fourth and final section of the book covers the application of molecular cytogenetic techniques to cancer diagnosis. Chapters include the use of CGH in cancer investigations and the application of interphase FISH for the BCR/ABL rearrangements in CML and for HER2 amplification in breast cancer. Also included in this section are the interesting combined approaches, firstly of chromogenic in situ hybridisation with FISH in pathology and secondly simultaneous fluorescence immunophenotyping and FISH on tumour cells.

With any multiauthor book, there are bound to be differences in approach to the writing of individual contributions. This book has taken a surprisingly consistent approach, perhaps an illustration of good editorial control. All of the chapters have good introductory sections and are well referenced, as well as containing the authors' preferred methodologies. Each chapter also includes a comprehensive notes section (effectively tips and troubleshooting advice from the experts). However, a major problem with the book is the lack of comparison between different molecular techniques. In the preface, it is suggested that the book should help the technologist or cytogeneticist determine which procedure to perform for an informative result. I am not sure that the book provides this, as most if not all of the contributors are bound to select their own preferred methodology. For example, in the chapter on FISH screening for telomere abnormalities, an otherwise excellent chapter by Samantha Knight and Jonathan Flint, the practical application of only one of the two commercially available probe sets for this test is covered. More importantly, elsewhere in the book there is a first class chapter on interphase FISH for prenatal diagnosis of common aneuploidies by Baruch Feldman *et al.* This covers the topic extremely comprehensively and provides 89 literature citations. However,

there is no mention, as far as I could see, throughout the book of the other alternative molecular approach of quantitative fluorescence PCR for prenatal aneuploidy detection. While it may be reasonable to excuse the editor by saying that the book is not designed to cover purely molecular genetic techniques, in the same section as the prenatal FISH, there is a chapter on the molecular detection of uniparental disomy. This chapter sits rather incongruously among the others, but is in itself a useful and important topic. Another chapter which seems to have lost its place describes the BAC resource for molecular cytogenetics. This is the final chapter in the book, which appears to have been added as an afterthought. Surely this should have been in evolving techniques and applications rather than "special applications in oncology". The oncology applications would also have benefited from more chapters, for example, haematological disorders other than CML, solid tumour FISH (other than HER2), and perhaps a chapter on the screening of urine samples for bladder cancer.

Perhaps these topics are covered in the companion volume (Methods in molecular biology, volume 220. Cancer cytogenetics: methods and protocols). Another criticism is that the provision of colour plates is very variable. Some chapters are well illustrated, others less so. For example, the chapter on SKY FISH relies on black and white illustrations, whereas the MFISH chapter has glorious full colour images. Furthermore, the overall size of the book is relatively small (16 cm by 24 cm) and the size of typesetting and tight layout does not make for easy reading when compared with, for example, Rooney's "Human cytogenetics: a practical approach".

On the plus side, there is an incredibly large amount of information packed into this volume, none of it superfluous. Although other textbooks that cover FISH techniques are available, this book provides a more comprehensive, up to date, and thorough coverage of diagnostic molecular cytogenetics than any of the other books currently available.

In summary, I would recommend it as a reference source for everyone working in and interested in the exciting field of diagnostic and research molecular cytogenetics.

Lionel Willatt

NOTICE

European Human Genetics Conference 3 – 6 May 2003 ICC, Birmingham, England

Plenary sessions

- Low Penetrance Genes and Cancer Susceptibility
- Public Issues - Population DNA Banks
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Symposia

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- Alternative splicing
- Cancer genetics
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- SNPs and haplotypes
- Chromosomes in genetic disease
- Genetics and endocrine problems

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- Syndrome identification
- Cytogenetics
- Problems in counselling/ethics
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- Prenatal cytogenetics
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Abstract deadline

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Further information available from: The Vienna Medical Academy of Postgraduate Medical Education and Research, Alserstrasse 4, A-1090 Vienna, Austria. Tel +43 1 405 13 83 22 Email: eshg@medacad.org

CORRECTIONS

In the October 2002 issue of the journal, in the paper by Van Maldergem *et al* (*J Med Genet* 2002;**39**:722-33), we regret that some of the authors' names and affiliations were inadvertently omitted. They were:

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In the July 2002 issue of the journal, in the Online mutation report by Olivieri *et al* (p e39), there was a misspelling in table 2. For No 12 the mutation should have been 1031G>T instead of 1006G>T.

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In the December 2002 issue of the journal, in the paper by Khoo *et al* (pp 906–912), all c.1732delTCinsAC mutations should read C.1732delTCinsA. This error occurs on page 906 (Abstract) and page 910 (Discussion).