Hereditary Hemorrhagic Telangiectasia: Breakpoint Characterization of a Novel Large Deletion in *ACVRL1* Suggests the Causing Mechanism

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Key Words

ACVRL1 · Large deletion · MLPA · Short direct repeats · Slippage

Abstract

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominant vascular dysplasia. Mutations in either *ENG* or *ACVRL1* account for around 85% of cases, and 10% are large deletions and duplications. Here we present a large novel deletion in *ACVRL1* gene and its molecular characterization in a 3 generation Italian family. We employed short tandem repeats (STRs) analysis, direct sequencing, multiplex ligation-dependant probe amplification (MLPA) analysis, and 'deletion-specific' PCR methods. STRs Analysis at *ENG* and *ACVRL1* loci suggested a positive linkage for *ACVRL1*. Direct sequencing of this gene did not identify any mutations, while MLPA identified a large deletion. These results were confirmed and exactly characterized with a 'deletion-specific' PCR: the deletion size is 4,594 bp and breakpoints in exon 3 and intron 8 show the presence of short direct repeats of 7 bp [GCCCCAC]. We hypothesize, as causative molecular mechanism, the replication slippage model. Understanding the fine mechanisms associated with genomic rearrange-

ments may indicate the nonrandomness of these events, highlighting hot spots regions. The complete concordance among MLPA, STRs analysis and 'deletion-specific PCR' supports the usefulness of MLPA in HHT molecular analysis.

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Hereditary hemorrhagic telangiectasia (HHT) or Rendu-Osler-Weber disease is an autosomal dominant vascular dysplasia that affects 1 in 5–8,000 [Lesca et al., 2007; Govani and Shovlin, 2009; Shovlin, 2010; Faughnan et al., 2011]. A clinical diagnosis of HHT is based on the presence of at least 3 of the following 'Curaçao criteria': (1) spontaneous, recurrent epistaxis; (2) multiple telangiectases at characteristic sites as nose, lips, oral cavity, and fingers tips; (3) visceral lesions, such as arteriovenous malformations in lungs, liver, brain, spinal cord, and GI tract; (4) family history of a first-degree relative with HHT who has been diagnosed with the same criteria [Shovlin et al., 2000]. HHT presentation patterns are highly variable even within families [Govani and Shovlin, 2009]. HHT patients carry mutations in *ENG* (OMIM 131195) (HHT type 1: OMIM 187300), *ACVRL1* (OMIM 601284) (HHT type 2: OMIM 600376) or *SMAD4* (OMIM

Fig. 1. The pedigree of the family. The proband (II:1) and all analysed affected family members (black-shaded) were positive for the *ACVRL1* exon 3–8 deletion. MLPA results are indicated for each family member as (+) or (–) for the presence or absence of the *ACVRL1* deletion, respectively. D12S1677 is an intragenic marker located in IVS9.

600973) (HHT in association with juvenile polyposis (JPHT): OMIM 175050) genes [McAllister et al., 1994; Johnson et al., 1996; Gallione et al., 2004].

All these genes belong to the TGF β /BMPs pathway [David et al., 2007]. Two additional HHT loci have been reported, but no new disease causing gene has been identified till now [Cole et al., 2005; Bayrak-Toydemir et al., 2006]. Screening for *ENG* and *ACVRL1* mutations by full coding region sequencing and analysis for copy number changes by multiplex ligation-dependent probe amplification (MLPA) or quantitative multiplex-polymerase chain reaction (QM-PCR) identifies mutations for approximately 80–90% of clinically diagnosed patients [McDonald et al., 2011]. Until now, over 350 mutations in *ACVRL1* and 450 mutations in *ENG* have been described in the HHT mutation database (<http://arup.utah.edu/database/HHT/>). These mutations, most of which are private, include missense, nonsense, splice site mutations, small deletions or duplications, and insertions [Lesca et al., 2008]. Approximately 10% of HHT patients have large deletions or duplications [Lesca et al., 2006; Prigoda et al.,

2006; Richards-Yutz et al., 2010; McDonald et al., 2011]. In a few cases, the breakpoints of the rearrangements and the mechanism of origin of the abnormality have been studied [Prigoda et al., 2006; Shoukier et al., 2008; Wooderchak et al., 2010]. These kind of mutations are generally missed by standard PCR amplification and direct sequencing. Here, we report on a novel large deletion in *ACVRL1* gene and its molecular characterization in a large Italian family with clinically confirmed HHT.

Materials and Methods

Blood samples from 16 members (11 affected) of a large HHT Italian family diagnosed according to Curaçao criteria were collected by E.B. A written informed consent was obtained from all subjects. Genomic DNA was extracted from peripheral EDTA-anticoagulated blood samples using standard procedures. Linkage analysis for the *ENG* locus on chromosome 9 (about 15 cM) and *ACVRL1* locus on chromosome 12 (about 10 cM) was performed using 6 and 5 short tandem repeat (STR) markers, respectively (STRs *ENG*: available on request; STRs *ACVRL1*: see fig.1). Primer

sequences were found in NCBI UniSTS database (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=unists>) and forward primers were conjugated with Hex or 6-FAM fluorochrome. Results were analyzed and visualized with Peak Scanner (Applied Biosystems, Foster City, Calif., USA). All coding exons and exon-intron boundaries of *ACVRL1* gene (Ref Seq: NG_009549.1) were amplified according to Olivieri et al. [2007] and sequenced. The PCR products were sequenced with ABI PRISM 3730XL Genetic Analyser with Applera BigDye v3.1 sequencing kit (Applied Biosystems). We used the P093-B1 SALSA MLPA kit HHT/PPH1 (MRC-Holland, Amsterdam, The Netherlands) to analyze large deletions or duplications in *ACVRL1*, *ENG* and *BMPR2* genes according to the manufacturer's instructions. Probe amplification products were analyzed on an ABI PRISM 3100 Genetic Analyser using Liz500 size standard and G5 filter; MLPA peak plots were visualized with Peak Scanner (Applied Biosystems). Normalization and dosage ratios were calculated using Coffalyser (MRC-Holland). Limits of dosage ratios for deletions and duplications are ≤ 0.7 and ≥ 1.35 , respectively. We designed primer pairs for deletion-specific PCR using free available softwares: Primer3 (<http://frodo.wi.mit.edu/primer3/>) and Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). To resolve the 5' and 3' breakpoints, we performed a long-range PCR (denaturation at 94°C for 5 min, denaturation at 98°C for 1 min, annealing at 60°C for 1 min, elongation at 72°C for 6 min with the latter 3 steps repeated for 35 cycles and an additional elongation step at 72°C for 5 min) with primers in intron 2 and intron 8 using TaKaRa LA Taq (Takara Bio Inc., Japan) according to manufacturer's instructions. We screened for interspersed repeats in the *ACVRL1* locus and the region of the deletion using the software RepeatMasker (<http://www.repeatmasker.org/>).

Results

In our HHT family, linkage analysis identified a common haplotype for STR markers around the *ACVRL1* gene shared by all affected members (fig. 1), but sequencing of the gene coding region failed to identify any disease-causing mutations. Instead, MLPA identified a large deletion, spanning exons 3–8 of *ACVRL1*, in the index case (fig. 2a) and familial cosegregation of deletion and disease was then confirmed (fig. 1). With a 'deletion-specific PCR', we found a 4,594-bp deletion; deleted region coordinates on build Hg18 (NCBI 36) are: chr12:g.50593334–50597927 (NG_009549.1:g.10866_15459del) (fig. 2b). 5' breakpoint is located in exon 3, 19 bp upstream the MLPA exon 3 probe, and 3' breakpoint in intron 8. We obtained a fine definition of the sequences bordering the breakpoints using direct sequencing. This deletion can be defined as a 'submicroscopic rearrangement' or 'small genomic change' as its size is <5 Mb [Stankiewicz and Lupski, 2010]. We searched the deletion we found in the Database of Genomic Variants (DGV) (<http://www.projects.tcag.ca/variation/>), the HHT Mutation Database and the Human Gene Mutation Database (HGMD) (<http://www.hgmd.org/>).

Two deletions involving exons 3–8 of *ACVRL1* gene are present [Prigoda et al., 2006; Richards-Yutz et al., 2010]: the first one [Prigoda et al., 2006] was reported in 2 apparently unrelated families and breakpoint characterization demonstrated different positions both for 5' end (which fell in IVS2) and 3' end. Breakpoints of the second deletion [Richards-Yutz et al., 2010] were not characterized at a molecular level.

The analysis with RepeatMasker of the *ACVRL1* locus revealed a short interspersed element (SINE) density of 22.30% (Alu density of 8.82% and mammalian interspersed repeat (MIR) density of 13.47%) and a total interspersed repeats density (SINE + LINE) of 24.74%. Within the region of our deletion, we found only SINEs with a density of 24.29% (Alu density of 12.89% and MIR density of 11.41%). The C + G content of *ACVRL1* locus is 54.28% and in the deleted region 56.51%.

Discussion

Standard molecular genetic testing of HHT includes sequencing of the coding regions and analysis for copy number changes for *ENG* and *ACVRL1* genes [Lesca et al., 2006; Prigoda et al., 2006; McDonald et al., 2011]. In our family, STRs analysis at *ENG* and *ACVRL1* loci showed a common haplotype for the *ACVRL1* gene in all affected members. Performing MLPA was necessary because the sequence analysis in this gene did not identify any mutations. We found a large deletion that we decided to characterize using a 'deletion-specific PCR'. The 4,594 bp deletion we found results in loss of the intervening sequence between 2 perfect direct 7-bp repeats [GCCCCAC] and elimination of 1 of these repeats (see fig. 2). This sequence includes the 'immunoglobulin heavy chain class switch repeat', a short direct repeat of 5 bp [CCCCA] [Chen et al., 2005a, b], which has been reported to mediate slippage mispairing and to cause deletions and translocations [Demura et al., 2002; Abeyasinghe et al., 2003]. It is well documented that the slippage mechanism during replication process is mediated by the presence of direct repeats (2–8 bp) at the end points of deletions/duplications and that only one short repeat is retained in the rearrangement [Ketterling et al., 1994; Chen et al., 2005a, b; Taulan et al., 2009]. Several characterized deletions showed locus variability both in the sequence and length of the direct repeats. Probably other factors, such as chromatin higher-order structure [Woodcock and Ghosh, 2010] and chromosomal location of the loci may play an important role in generating these rearrangements [Tau-

Fig. 2. Characterization of the deletion. **a** MLPA results obtained with Coffalyser. The ACVRL1 deleted exons 3–8 have a peak ratio <0.7. **b** Schematization of the deleted region and sequence of the PCR product encompassing the breakpoints and showing a deletion of 4,594 bp (Hg18 (NCBI 36): chr12:g.50593334–50597927).

Black vertical arrows in the scheme indicate deletion breakpoints. White dashed vertical lines indicate MLPA probes position. Large horizontal arrows indicate position of primers for deletion-specific PCR. In the electropherogram, the 'immunoglobulin heavy chain class switch repeat' sequence is boxed.

lan et al., 2009]. Interestingly, using RepeatMasker we found 24.74% of total interspersed repeats for the *ACVRL1* gene, which could cause instability of the region [Resta et al., 2010]. Moreover, the deleted region contains 2 MIRc repeats and 5 MIR repeats and this kind of internal motifs might fold into secondary DNA conformations that could have a role in the slippage model [Bacolla et al., 2006]. Lesca et al. [2006] underlined that the density of *Alu* repeats that can lead to unequal recombination is higher in the *ENG* gene than in *ACVRL1*, and it can explain the higher frequencies of large deletions or duplications in *ENG*. McDonald et al. [2011] also suggested that repetitive elements would predispose recombination and genomic rearrangements in *ENG*. A detailed definition of large deletions was reported by Wooderchak et al. [2010], who found a dynamic deletion in *ENG* and Shoukier et al. [2008], who described 1 deletion in *ENG* and 4 deletions involving *ACVRL1*. They suggest non-homologous end joining as the probable mechanism of origin of these deletions because there was little homology between the breakpoint flanking sequences. In this work, we characterized the deletion breakpoints in the *ACVRL1* gene and also hypothesized the slippage mispairing as the probable causing mechanism. Understanding the fine mechanisms associated with genomic rearrangements may indicate the nonrandomness of these events, highlighting hot

spots regions able to mediate rearrangements [Eyries et al., 2011]. MLPA is a new approach for the detection of genomic rearrangements. This technique has proven to be a useful and reliable diagnostic tool in several genetic disorders [Paracchini et al., 2008; Wooderchak-Donahue et al., 2011]. In HHT molecular analysis, MLPA has been reported to be used on only a small number of samples, thus with limited information about its sensitivity [Shoukier et al., 2008; Wooderchak et al., 2010; McDonald et al., 2011]. For this reason, any rearrangements found with MLPA must be interpreted carefully and verified by alternative techniques, such as 'deletion-specific PCR'.

The complete concordance among MLPA, STRs analysis and 'deletion-specific PCR' for the family tested supports MLPA as an efficient technique. Our results suggest the 'slippage mispairing' as the likely mechanism for the origin of the deletion we studied and emphasize the importance of screening for large rearrangements in HHT patients negative to sequence analysis or in presence of variants with uncertain pathogenicity in *ACVRL1* and *ENG* genes.

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