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

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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 rearrangements 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.

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 600376).
(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 TGFβ/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; 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).
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/prim- er3/) 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/varia- tion/), 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 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 [CCCCCA] [Chen et al., 2005a, b], which has been reported to mediate slippage mispairing and to cause deletions and translocations [Demura et al., 2002; Abeyesinghe 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.
Characterization of a Novel ACVRL1 Large Deletion

References


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