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Germline mutations in *BMP9* are not identified in a series of Danish and French patients with hereditary hemorrhagic telangiectasia

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ABSTRACT

Hereditary hemorrhagic telangiectasia (HHT), an inherited vascular disorder, is, in the majority of cases (85%), caused by mutations in one of three genes (*ENG*, *ACVRL1*, and *SMAD4*). In the remaining group of individuals with clinical HHT, mutations have not been identified, suggesting yet undiscovered HHT causative genes.
A new vascular-anomaly syndrome caused by mutations in *BMP9* has recently been published. Three patients suspected of HHT, with familial nose bleedings and dermal manifestations not characteristic for HHT, were described. Although, it was concluded that these patients probably had a different vascular-anomaly syndrome, the suspicion that *BMP9* mutations might cause HHT remained.
To evaluate if germline mutations in *BMP9* can be identified in HHT patients, we investigated the Danish and the French Lyon cohort of mutation-negative and clinically definite HHT patients.
Exons and exon-intron boundaries of *BMP9* were analyzed by bi-directional Sanger sequencing in 28 clinical HHT patients (from 28 different families) with no pathogenic mutations in *ENG*, *ACVRL1* or *SMAD4*. No mutations of potential pathogenicity were identified in BMP9.
This study does not suggest that *BMP9* mutations causes HHT, although this cannot be fully excluded based on this study. So far, we have been unable to identify any patients with clinical HHT caused by a *BMP9* mutation.

1. Introduction

Hereditary hemorrhagic telangiectasia (HHT), also known as Osler-Weber-Rendu disease, is an autosomal dominantly inherited vascular disorder characterized by the presence of mucocutaneous telangiectases and arteriovenous malformations (AVM) in visceral organs, primarily the lungs, brain and liver. HHT is caused by mutations in three known genes of the transforming growth factor beta (TGF-β) signaling pathway: *ENG* (MIM 131195) (McAllister et al., 1994), *ACVRL1* (MIM 601284) (Johnson et al., 1995) and *SMAD4* (MIM 600993) (Gallione et al., 2004). Approximately 85% of HHT patients (Brusgaard et al., 2004; Schulte et al., 2005; Lesca et al., 2004) have a mutation identified in either *ENG* or *ACVRL1*. A mixed syndrome consisting of HHT and Juvenile Polyposis caused by mutations in the *SMAD4* gene represents approximately 2% of the HHT families (Gallione et al., 2004; Gallione et al., 2006). Nonetheless, in around 15% of individuals with definite clinical HHT a disease causing mutation remains unidentified, suggesting the existence of other undiscovered HHT causing genes. Two other loci on chromosome 5q31 (Cole et al., 2005) and 7p14 (Bayraktoydemir et al., 2006) have been linked to HHT, but the corresponding genes are not known yet. Identification of further causative genes will help our understanding of the molecular basis of HHT, and provide the possibility of identifying causative mutations in an additional number of HHT families.

The clinical diagnosis of HHT is based on the four Curaçao criteria (Shovlin et al., 2000): spontaneous and recurrent epistaxis; telangiectases at characteristic sites (lips, oral cavity, fingers and nose); visceral AVMs; and a first-degree relative with HHT. Fulfilling at least three criteria make a definite diagnosis of HHT, two criteria are considered possible HHT, and one or no criteria make HHT unlikely. van Gent et al. (2013) showed that the positive predictive value of a definite clinical diagnosis, based on the Curaçao criteria, was 100% using genetic testing as the gold standard.

Abbreviation: HHT, hereditary hemorrhagic telangiectasia.

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Wooderchak-Donahue et al. (2013) described a vascular anomaly syndrome with phenotypic overlap with HHT caused by *BMP9* mutations. Heterozygous *BMP9* missense mutations were identified in blood samples from three probands initially suspected of HHT or at least referred for genetic testing of HHT. These three individuals had epistaxis and cutaneous lesions that were described as telangiectases, but whose anatomical location and appearance were not characteristic of HHT, as some of them presented more diffusely and with location on the limbs and chest. Based on their clinical findings, the authors concluded that the patients did probably not have HHT, but a new syndrome with a similar and overlapping phenotype.

BMP9 (Bone Morphogenetic Protein 9) (MIM 605120), also known as *GDF2* (Growth/Differentiation Factor 2), is a member of the highly conserved TGF-β superfamily, and the authors hypothesized that since *BMP9* is located upstream from *ENG* and *ACVRL1* in the pathway, it is likely to cause a vascular-malformation syndrome with phenotypic overlap with HHT.

However, the suspicion that *BMP9* might also cause Curaçao definite HHT still remained and the Human Gene Mutation Database (HGMD®) (www.hgmd.org) and NCBI (National Center of Biotechnology Information) currently list *BMP9* as an HHT causing gene (www.ncbi.nlm.nih.gov/gene/2658; http://omim.org/entry/615506).

The purpose of this study was to investigate whether germline *BMP9* mutations can be identified in clinical HHT patients. We tested mutation-negative patients from the clinically well-described Danish cohort of HHT patients, as well as the mutation-negative patients from the French Lyon cohort, for germline mutations in *BMP9*. As all the patients in this study fulfil the Curacao criteria and thereby have definite HHT, performing mutation analysis of *BMP9* in this group, could potentially reveal if *BMP9* is in fact a novel HHT causative gene.

2. Materials and methods

In this study we included Danish and French HHT patients with definite clinical HHT according to the Curaçao criteria (Shovlin et al., 2000), but without a located disease causing mutation in *ENG*, *ACVRL1* or *SMAD4*.

2.1. Danish patients

They were part of the Danish HHT cohort of 107 families with a mutation detection rate of 89% (Torring et al., 2014). A total of 14 mutation-negative families were identified, of which 12 were previously published by Torring et al. (Torring et al., 2014). All HHT probands were initially evaluated at the national HHT Centre in Denmark. As previously described, all patients were offered screening for pulmonary arteriovenous malformations (PAVM), and screened for mutations in *ENG*, *ACVRL1* and *SMAD4* (Torring et al., 2014).

2.2. French patients

They were part of the French HHT Reference Center cohort (Lyon center) of 580 families with a mutation detection rate of 86%. However, all clinical data on the mutation-negative patients were recently reviewed, and of these, 14 families had clinically definite HHT. All HHT probands were initially evaluated at the national HHT Centre in France. As previously described, all patients were offered screening for pulmonary (CT scan) and hepatic (liver ultrasound) arteriovenous malformations, and screened for mutations in *ENG*, *ACVRL1* and *SMAD4* (Lesca et al., 2004).

2.3. Mutation analysis of the ENG, ACVRL1, and SMAD4 genes

All exons and exon-intron boundaries of *ENG* (RefSeq: NM_001114753.1), *ACVRL1* (RefSeq: NM_000020.2), and *SMAD4* (RefSeq: NM_005359.5) were analyzed by bi-directional Sanger sequencing (Torring et al., 2014). The Salsa Multiplex Ligation-dependent Probe Amplification (MLPA) probemix kits P093 HHT/PPHI and P158-C2 JPS (MRC Holland BV, Amsterdam, The Netherlands) were used to screen for rearrangements of respectively the *ENG*, *ACVRL1*, and *SMAD4* genes, according to the manufacturer's standard protocol.

The phenotype of each patient is listed in Table 1.

2.4. Ethics statement

The patient consented to have HHT-genotyping performed after verbal information. The Danish patients consented in a clinical setting and according to Danish standards oral consent is sufficient. Written consent was obtained for French patients.

The Danish HHT Database was approved by the Danish Data Protection Agency (j.nr.2011-41-5962), and The local data protection agency 2008-58-0035.

The French database was approved by the "Institut National de Veille Sanitaire".

2.5. Mutation analysis of the BMP9 gene

Genomic DNA was isolated from peripheral leukocytes. Exons and exon-intron boundaries of *BMP9* (*GDF2*) (RefSeq: NM_016204.1) were analyzed by bi-directional Sanger sequencing using the BigDye® Terminator v.3.1 cycle sequencing kit (Applied Biosystems) and an ABI3730XL capillary sequencer (Applied Biosystems). The identified variants were named according to the international recommendations for nomenclature (www.hgvs.org/mutnomen/).

2.6. Evaluation of findings

Human Gene Mutation Database (HGMD®) (www.hgmd.org/), dbSNP (www.ncbi.nlm.nih.gov/SNP/), and the Exome Variant Server (http://evs.gs.washington.edu/EVS/) were consulted to detect if the identified variants were previously described and to identify known single nucleotide polymorphisms. For splice variants, the pathogenicity was estimated based on five splice site prediction programs [SpliceSiteFinder-like (Zhang, 1998), MaxEntScan (Yeo and Burge, 2004), GeneSplicer (Pertea et al., 2001), NNSPLICE (Reese et al., 1997) and Human Splicing Finder (Desmet et al., 2009)] using AlaMut version 2.3 (Interactive Biosoftware, Rouen, France).

3. Results

3.1. Genetic testing

We analyzed exons and exon-intron boundaries of the *BMP9* gene and identified four different well known single nucleotide polymorphisms (rs7923671, rs12252199, rs9971293 and rs3781226) in several cases. In the proband of family 142 we identified a splice site variant (c.-12C>A, p.?) which is reported in ExAC (http://exac.broadinstitute.org) with a frequency of 1:19,830 and must be considered a rare benign variant. Furthermore, all five splice site prediction programs predicted the splice variant to be of no significance and therefore benign.

No clear pathogenic or suspected pathogenic variant was identified.

4. Discussion

In the recently published paper by Wooderchak-Donahue et al. (2013), heterozygous *BMP9* mutations were identified in blood samples from three probands initially suspected of HHT. These three individuals were 14, 37 and 38 years old and all had recurrent epistaxis and a family history of siblings and/or parents with epistaxis. No screening for solid-organ AVMs was performed, except for brain-MRI in one individual which did not show any AVMs. The cutaneous lesions of the three patients tended to differ from what is characteristic of HHT: individual 1