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Research paper

Functional analysis of a novel *ENG* variant in a patient with hereditary hemorrhagic telangiectasia (HHT) identifies a new Sp1 binding-site

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ABSTRACT

Hereditary Hemorrhagic Telangiectasia (HHT) is a rare disease, with an autosomal dominant inheritance and a worldwide incidence of about 1: 5000 individuals. In > 80% of patients, HHT is caused by mutations in either *ENG* or *ACVRL1*, which code for ENDOGLIN and Activin A Receptor Type II-Like Kinase 1 (ALK1), belonging to the TGF-β/BMP signalling pathway. Typical HHT clinical features are mucocutaneous telangiectases, arteriovenous malformations, spontaneous and recurrent epistaxis, as well as gastrointestinal bleedings. An additional, but less frequent, clinical manifestation in some HHT patients is the presence of Pulmonary Arterial Hypertension (PAH).

The aim of this work is to describe the functional role of a novel *ENG* intronic variant found in a patient affected by both HHT and PAH, in order to assess whether it has a pathogenic role. We proved that the variant lies in a novel binding-site for the transcription factor Sp1, known to be involved in the regulation of *ENG* and *ACVRL1* transcription. We confirmed a pathogenic role for this intronic variant, as it significantly reduces *ENG* transcription by affecting this novel Sp1 binding-site.

1. Introduction

Hereditary Hemorrhagic Telangiectasia (HHT) is a rare genetic disease, also known as Rendu-Osler-Weber Syndrome (ROW), with an autosomal dominant inheritance.

HHT affects approximately 1:5000 individuals worldwide. The main feature of HHT is the presence of abnormal vascular structures such as telangiectases and ArterioVenous Malformations (AVMs), defined as direct connections between arteries and veins, with the loss of the interposed capillary beds (McDonald et al., 2015; Shovlin, 2010). HHT-related telangiectases are small and arise at mucocutaneous sites, such as nasal and oral mucosae, the lips, upper fingertips and the gastrointestinal tract. AVMs are larger lesions that typically affect solid organs including the lungs (Pulmonary AVMs – PAVM), liver (Hepatic AVMs –

Abbreviations: Ab, antibody; *ACTB*, β-actin; *ACVRL1*, Activin A Receptor type II-Like Kinase 1; AVM, ArterioVenous Malformation; BMP, Bone Morphogenetic Protein; *BMPR2*, Bone Morphogenetic Protein Receptor Type 2; CAVM, Cerebral ArterioVenous Malformation; CTRL, control; dbSNP, Single Nucleotide Polymorphism database; DMEM, Dulbecco's Modified Eagle Medium; EMSA, Electrophoretic Mobility Shift Assay; *ENG*, ENDOGLIN; ExAC, Exome Aggregation Consortium; FCS, Fetal Calf Serum; HAVM, Hepatic ArterioVenous Malformation; HEK293T, Human Embryonic Kidney 293 T cells; HHT, Hereditary Hemorrhagic Telangiectasia; HPAH, Heritable Pulmonary Arterial Hypertension; IPAH, Idiopathic Pulmonary Arterial Hypertension; JP, Juvenile Polyposis; *MADH4*, Mothers Against Decapentaplegic Homolog 4; mPAP, mean Pulmonary Artery Pressure; MUT, mutated; NE, Nuclear Extract; PAH, Pulmonary Arterial Hypertension; PAVM, Pulmonary ArterioVenous Malformation; PBMCs, Peripheral Blood Mononuclear Cells; PH, Pulmonary Hypertension; PVR, Pulmonary Vascular Resistance; ROW, Rendu-Osler-Weber Syndrome; SCAVM, Spinal Cord ArterioVenous Malformation; TGF-β, Transforming Growth Factor beta; VUS, Variant of Uncertain Significance; WT, wild type

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HAVMs), brain (Cerebral AVMs – CAVMs) and spinal cord (Spinal Cord AVMs – SCAVMs) (Bayrak-Toydemir et al., 2004; Shovlin, 2010). Both telangiectases and AVMs are characterized by thin walls of the vessels, which are prone to bleed when exposed to high perfusion pressure (Govani and Shovlin, 2009). In fact, as a consequence of the nasal telangiectases rupture, chronic epistaxes are observed in almost all HHT patients after the age of 40 (Lesca et al., 2007).

HHT clinical diagnosis is definite when three out of the following four "Curaçao criteria" are observed in patients: i) spontaneous and recurrent epistaxes; ii) telangiectases at the characteristic sites; iii) AVMs in the above-mentioned internal organs and iv) a positive family history, *i.e.* a first degree relative with an HHT diagnosis based on the same criteria (Shovlin et al., 2000).

HHT was initially described as a familial disorder characterized by severe and recurrent nosebleeds, gastrointestinal bleedings and anaemia (Osler, 1901; Rendu, 1896; Weber, 1907). Today, the phenotypic spectrum of the disease has been expanded to also include Juvenile Polyposis (JP) (Gallione et al., 2004) and Pulmonary Arterial Hypertension (PAH) (Trembath et al., 2001). In particular, PAH (MIM#178600) is a subtype of Pulmonary Hypertension (PH) characterized by the presence of pre-capillary PH, demonstrated by a mean Pulmonary Artery Pressure (mPAP) ≥ 25 mmHg, a pulmonary artery wedge pressure ≤ 15 mmHg and Pulmonary Vascular Resistance (PVR) > 3 Wood units, evaluated by right heart catheterization, in the absence of other causes (Galiè et al., 2016). The term PAH includes different disease subtypes: idiopathic PAH (IPAH), heritable PAH (HPAH) and PAH associated with other systemic diseases or drug/toxin exposures (Austin and Loyd, 2013). Right heart catheterization remains essential for PH or PAH diagnosis (Galiè et al., 2016).

The genetic bases of HHT lie in the TGF-β/BMP signalling pathway, which is involved in the regulation of several physiological processes, including angiogenesis. To date, four genes encoding for proteins belonging to this pathway have been related to HHT. Mutations in *ENG* (MIM*131195), coding for ENDOGLIN (ENG), and *ACVRL1* (MIM *601284), coding for Activin A Receptor Type II-Like Kinase 1 (ALK1), are associated with HHT type 1 (MIM#187300) and type 2 (MIM#600376), respectively, and account for > 80% of patients (Olivieri et al., 2007). Mutations in *MADH4* (MIM*600993), coding for Mothers Against Decapentaplegic Homolog 4 (SMAD4), are observed in 2–3% of patients with a syndrome called JPHT (MIM#175050), characterized by both HHT and JP symptoms (Gallione et al., 2010). Lastly, mutations in *GDF2* (MIM *605120), coding for Bone Morphogenetic Protein 9 (BMP9), are described in only a few families and are associated with an HHT-like syndrome named HHT5 (MIM#615506) (Wooderchak-Donahue et al., 2013). Thus, in about 7–13% of individuals with an HHT clinical definite diagnosis, mutations in the previously listed genes are not found.

The development of PAH is related to the dysregulation of the TGF-β/BMP signalling pathway. In fact, mutations in *BMPR2* (Bone Morphogenetic Protein Receptor Type 2 – MIM*600799), encoding a receptor of the BMPs family, account for 75% of HPAH cases and for 15% of apparent IPAH cases (Austin and Loyd, 2013; West et al., 2014).

Here we present data about the pathogenic role of a novel *ENG* intronic Variant of Uncertain Significance (VUS), c.1852 + 42 C > T, observed in a patient with a PAH diagnosis followed by the identification of typical HHT clinical features. Thanks to different functional studies, we proved a decrease of *ENG* transcription due to the presence of this variant. In particular, this VUS impairs a novel intronic binding-site for the transcription factor Sp1, which is already known to be involved in *ENG* and *ACVRL1* transcription (Albiñana et al., 2017; Botella et al., 2001; Garrido-Martin et al., 2010; Ríus et al., 1998).

2. Materials and methods

2.1. Patients

Patients' blood samples were collected at the Cardiology Unit at IRCCS Fondazione Policlinico "San Matteo" in Pavia. Each patient had a complete cardiologic examination and signed an informed consent for molecular analyses. The study was performed in accordance with the ethical principles of the Declaration of Helsinki (http://www.wma.net/en/30publications/10policies/b3/).

2.2. DNA extraction, polimerase chain reaction (PCR) and multiplex ligation probe amplification (MLPA)

Genomic DNA was extracted from peripheral blood using the "GenElute™ Blood Genomic DNA Kit" by SIGMA, according to manufacturer's instructions. We used the online software "Primer 3" (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to design the *BMPR2*, *ENG* and *ACVRL1* primers used to amplify coding exons and flanking introns. The dimensions of the flanking intron sequences were evaluated individually for each exon to cover all regions that may potentially include known variants, either pathogenic or benign (http://arup.utah.edu/database/HHT/). The PCR, purified using MultiScreen®$PCR_{\mu 96}$ Filter Plate (Merck Millipore), underwent Sanger sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem™), following the manufacturer's instructions.

MLPA of *ENG*, *ACVRL1* and *BMPR2* was performed to assess the presence of large deletions or duplications, using the SALSA MLPA probemix P093-C2 HHT/PPH1 (MRC-Holland).

Both Sanger sequencing and fragment separation analyses were performed on a 3500 series Genetic Analyzer 8-Capillary (Applied Biosystem™). Regarding MLPA, a GS500 size standard was adopted and the results were analysed using Coffalyser software (https://coffalyser.wordpress.com/).

2.3. Plasmids site-directed mutagenesis

Site-directed mutagenesis was performed on two constructs: pcEXV-ENG (Bellón et al., 1993) and pDisplay-MinigEND (Blanco and Bernabeu, 2011). The first plasmid contains the entire *ENG* coding sequence and intron 14, while the second one contains the *ENG* sequence from the beginning of exon 14 to the end of exon 15. The mutagenesis was carried out using primers specifically designed with the variant of interest (Forward: 5′– GGGCCCCT**T**CATCCACCC – 3′ and Reverse: 5′– GGGTGGATG**A**AGGGGCCC – 3′; in bold the nucleotide change).

2.4. Bacterial transformation and cell transfection

Mutated constructs were used to transform One Shot TOP10 Chemically Competent *E. coli*. Bacteria were grown in LB agar plates (100 μg/mL ampicillin). Single colonies were inoculated in LB medium (100 μg/mL ampicillin) and incubated overnight at 37 °C. DNA was extracted from bacteria by using PureYield™ Plasmid Miniprep System (Promega). The *ENG* insert of both constructs was fully sequenced and the mutation, as well as the absence of any error introduced by the amplification, was verified.

Mutated constructs were used to transfect Human Embryonic Kidney 293 T (HEK293T) cells, grown in Dulbecco's Modified Eagle Medium (DMEM - Gibco) supplemented by 10% heat-inactivated Fetal Calf Serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin. Cell transfection was carried out using Lipofectamine® LTX Reagent (Invitrogen), following the manufacturer's instructions.