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Global gene expression profiling of telangiectasial tissue from patients with hereditary hemorrhagic telangiectasia

Pernille M. Tørring [a,c,*], Martin Jakob Larsen [a,d], Anette D. Kjeldsen [b,c], Lilian Bomme Ousager [a,d], Qihua Tan [a,e], Klaus Brusgaard [a,d]

[a] Department of Clinical Genetics, Odense University Hospital, Denmark
[b] Department of Otorhinolaryngology, Odense University Hospital, Denmark
[c] Otorhinolaryngology, Institute of Clinical Research, University of Southern Denmark, Denmark
[d] Human Genetics, Institute of Clinical Research, University of Southern Denmark, Denmark
[e] Epidemiology, Biostatistics and Biodemography, Institute of Public Health, University of Southern Denmark, Denmark

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ABSTRACT

Hereditary hemorrhagic telangiectasia (HHT), the most common inherited vascular disorder, is predominantly caused by mutations in *ENG* and *ACVRL1*, which are part of the transforming growth factor beta (TGF-β) signaling pathway. HHT is characterized by the presence of mucocutaneous telangiectases and arteriovenous malformations in visceral organs, primarily the lungs, brain and liver. The most common symptom in HHT is epistaxis originating from nasal telangiectasia, which can be difficult to prevent and can lead to severe anemia. The clinical manifestations of HHT are extremely variable, even within family members, and the exact mechanism of how endoglin and ALK1 haploinsufficiency leads to HHT manifestations remains to be identified.

Objectives: The purpose of this study was to detect significantly differentially regulated genes in HHT, and try to elucidate the pathways and regulatory mechanisms occurring in the affected tissue of HHT patients, in order to further characterize this disorder and hypothesize on how telangiectases develop.

By microarray technology (Agilent G3 Human GE 8x60), we performed global gene expression profiling of mRNA transcripts from HHT nasal telangiectasial ($n = 40$) and non-telangiectasial ($n = 40$) tissue using a paired design. Comparing HHT telangiectasial and non-telangiectasial tissue, significantly differentially expressed genes were detected using a paired *t*-test. Gene set analysis was performed using GSA-SNP.

In the group of *ENG* mutation carriers, we detected 67 differentially expressed mRNAs, of which 62 were down-regulated in the telangiectasial tissue. Gene set analysis identified the gene ontology (GO) terms vasculogenesis, TGF-β signaling, and Wnt signaling as differentially expressed in HHT1. Altered Wnt signaling might be related to HHT pathogenesis and a greater understanding of this may lead to the discovery of therapeutic targets in HHT.

Introduction

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal dominantly inherited vascular disorder characterized by the presence of mucocutaneous telangiectasia and arteriovenous malformations (AVMs) in visceral organs, primarily the lungs, liver and brain. The most common clinical manifestation is spontaneous and recurrent epistaxis (Faughnan et al., 2011; Kjeldsen et al., 2000), originating from nasal telangiectasias, which can be difficult to prevent and can lead to severe anemia. Moreover, pulmonary arteriovenous malformations (AVMs) occur in approximately one third of patients and are potentially lethal due to hemorrhage or shunting of blood through these abnormal blood vessels, which can cause paradoxical embolisms and cerebral abscesses (Kjeldsen et al., 2000, 2014). The clinical expression of HHT is extremely variable, even between family members, and age-dependent penetrance is present. Around 85% of clinically diagnosed HHT patients carry a mutation in either *ENG* (HHT1) or *ACVRL1* (HHT2) (Brusgaard et al., 2004; Lesca et al., 2004; Schulte et al., 2005; Torring et al., 2014a). Patients with mutations in *ENG* or *ACVRL1* are clinically similar, as all reported manifestations are known to occur in both. However, a tendency towards later onset, fewer pulmonary and cerebral AVMs, more liver involvement, and a higher risk of developing pulmonary arterial hypertension are observed in patients with *ACVRL1* mutations (Faughnan et al., 2011). Currently there is no cure for the disease; only symptomatic treatment is possible.

HHT manifestations are thought to result from an imbalance in the process of angiogenesis. Angiogenesis is defined as the physiological process through which new blood vessels form from pre-existing

* Corresponding author at: Department of Clinical Genetics, Odense University Hospital, Sdr. Boulevard 29, DK-5000 Odense C, Denmark. Fax: +45 65414875.
E-mail address: pernille.toerring@rsyd.dk (P.M. Tørring).

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vessels, and is controlled by different cytokines such as vascular endothelial growth factor (VEGF) and transforming growth factor beta 1 (TGFβ1). *ENG* and *ACVRL1* encode the receptor proteins: endoglin and ALK1 (activin A receptor type II-like 1) (also known as SKR3) respectively, which are components of the TGF-β signaling pathway. The TGF-β signaling pathway plays a complex and important role in the development and homeostasis of many organs, including the vascular system, in which it is involved in cell proliferation, migration, extracellular matrix formation, vascular smooth muscle cell differentiation and vascular tone. As endoglin and ALK1 proteins are predominantly expressed in endothelial cells, these are primary cellular targets of the disease. Thus, HHT manifestations are caused by a disturbance in the TGF-β signaling pathway (Attisano and Wrana, 2000; Goumans et al., 2002; Lebrin et al., 2004). Protein expression studies have shown that endoglin and ALK1 haploinsufficiency, by mutations that cause loss of function, is the underlying cause of HHT (Pece-Barbara et al., 1999). How haploinsufficiency leads to HHT manifestations is, however, not yet identified. Gene expression analysis of HHT telangiectasial tissue could lead to important insight into the pathophysiology of HHT manifestations and into the pathways involved in the disease mechanisms.

In 2007, two gene expression microarray studies were published describing cultured HHT samples. Thomas et al. (2007) studied cultured human umbilical vein endothelial cells from seven newborns carrying the familial HHT mutation, using human umbilical vein endothelial cells from three healthy newborns as controls. They found an altered gene expression profile associated with HHT, which concerned genes involved in vasculogenesis, angiogenesis, extracellular matrix and cellular adhesion. Fernandez-L et al. (2007) studied cultured endothelial cells from three HHT patients using cultured endothelial cells from healthy donors as controls. They also found an altered gene expression profile, primarily affecting genes involved in angiogenesis, cytoskeleton, cell migration, proliferation and NO synthesis.

In contrast to the two studies performed using cultured endothelial cells, this study used non-cultured and affected HHT tissue in microarray gene expression profiling. By comparing telangiectasial and normal tissue from HHT patients our purpose was to detect significantly differentially regulated genes in HHT, and try to elucidate the pathways and regulatory mechanisms occurring in the affected tissue, in order to further characterize this disorder and hypothesize how telangiectases develop. More insight into the disease-causing mechanisms in HHT could potentially provide new treatment possibilities.

We identified differentially expressed genes in HHT telangiectasial tissue compared with non-telangiectasial tissue, using a paired design. Moreover, gene set analysis identified Wnt signaling as differentially expressed in HHT1.

Materials and methods

HHT patients and controls

Nasal mucosal biopsy specimens of telangiectasial and non-telangiectasial tissue in pairs from HHT1 ($n = 19$) and HHT2 ($n = 21$) patients were used in this study. Participants and tissue handling have been described in a previous study aimed at identifying differentially expressed long non-coding RNA in HHT (Torring et al., 2014b). Briefly described, the samples were collected using a Weil nasal forceps from the middle or inferior nasal concha and contained macroscopically visible telangiectases in one sample and macroscopic natural mucosa in the second sample. All patients showed signs of HHT according to the Curaçao Criteria (Shovlin, 2000) and carried the familial pathogenic mutation in either *ENG* or *ACVRL1* (Table 1) (Torring et al., 2014a).

Besides this paired design, we collected nasal mucosal samples from healthy siblings of the included HHT patients when possible. This resulted in 6 sibling samples from 6 separate HHT1 families and 9 sibling samples from 7 HHT2 families. The healthy siblings were aged 26–62 years (median age 45) and included 4 men and 11 women. The healthy siblings were all genetically tested and did not carry the familial mutation (Table 1).

This study was approved by the local ethics committee (S-20090131) and the participants provided written informed consent.

RNA isolation and microarray hybridization

Total RNA was isolated using the RNeasy Micro Kit according to the RNeasy® Micro Handbook (Qiagen) and sample labeling and array hybridization were performed according to the Two-Color Microarray-Based Gene Expression Analysis-Low Input Quick Amp Labeling-protocol (Agilent Technologies) using the SurePrint G3 Human Gene Expression 8x60 microarray format (Agilent Technologies) as previously described (Torring et al., 2014b).

Data pre-processing and annotation of mRNAs

Date pre-processing was performed as previously described (Torring et al., 2014b). Microarray data was deposited in the Gene Expression Omnibus (GSE53515).

All the 42,164 probes of the Agilent SurePrint G3 array were annotated using GENCODE v.16 gene annotation database (www.gencodegenes.org) (Derrien et al., 2012). The genomic coordinates of the probes in the Agilent array were matched to the genomic coordinates of the mRNAs from the GENCODE v.16, to identify the probes covering mRNAs, which were included if 55 base pairs overlapped with the 60-mer array probes. The resulting 25,568 probes mapping to 18,478 unique mRNAs were used for further analysis.

Statistical analysis

Statistical analyses were performed using the Qlucore Omics Explorer 3.0 software (Qlucore). Differentially expressed mRNAs, comparing telangiectasial and non-telangiectasial tissue, were ranked according to statistical significance determined by a paired *t*-test. Variance filtering was performed based on the projection score value (Fontes and Soneson, 2011). Analysis was done separately for the groups HHT1 and HHT2. Multiple testing was adjusted for by the Benjamini–Hochberg method. The results were considered statistically significant when $q < 0.05$.

Comparison of normal tissue from HHT mutation carriers with healthy siblings

Samples from healthy siblings [$n = 6$ (HHT1) and $n = 9$ (HHT2)] (Table 1) were compared with the HHT patients' non-telangiectasial samples, in order to evaluate the non-telangiectasial samples as controls in the paired design. Statistical analyses were performed using the Qlucore Omics Explorer 3.0 software (Qlucore). Differentially expressed mRNAs comparing non-telangiectasial HHT tissue and nasal tissue from healthy siblings were ranked according to statistical significance, determined by an unpaired *t*-test. Variance filtering was performed based on the projection score value. Analyses were performed separately for groups HHT1 and HHT2.

Clustering analysis

Hierarchical clustering and unsupervised principal component analysis (PCA) were performed in Qlucore Omics Explorer 3.0. Expression levels of each gene were standardized to zero mean and unit variance.

Gene set analysis

To explore functional biological differences between telangiectasial and non-telangiectasial tissues, the software GSA-SNP (gene set analysis-single nucleotide polymorphism; http://gsa-snp.sourceforge.