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Decreased levels of miR-28-5p and miR-361-3p and increased levels of insulin-like growth factor 1 mRNA in mononuclear cells from patients with hereditary hemorrhagic telangiectasia¹

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Abstract

Hereditary hemorrhagic telangiectasia (HHT) is a rare vascular disorder inherited in an autosomal dominant manner. Patients with HHT can develop vascular dysplasias called telangiectasias and arteriovenous malformations (AVMs). Our objective was to profile and characterize micro-RNAs (miRNAs), short noncoding RNAs that regulate gene expression posttranscription-ally, in HHT patient-derived peripheral blood mononuclear cells (PBMCs). PBMCs, comprised mostly of lymphocytes and monocytes, have been reported to be dysfunctional in HHT. A total of 40 clinically confirmed HHT patients and 22 controls were enrolled in this study. PBMCs were isolated from 16 mL of peripheral blood and purified for total RNA. MiRNA expression profiling was conducted with a human miRNA array analysis. Select dysregulated miRNAs and miRNA targets were validated with reverse transcription–quantitative polymerase chain reaction. Of the 377 miRNAs screened, 41 dysregulated miRNAs were identified. Both miR-28–5p and miR-361–3p, known to target insulin-like growth factor 1 (IGF1), a potent angiogenic growth factor, were found to be significantly downregulated in HHT patients. Consequently, IGF1 mRNA levels were found to be significantly elevated. Our research successfully identified miRNA dysregulation and

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Conflict of interest

The authors declare that there is no conflict of interest associated with this work.

elevated IGF1 mRNA levels in PBMCs from HHT patients. This novel discovery represents a potential pathogenic mechanism that could be targeted to alleviate clinical manifestations of HHT.

Résumé:

La télangiectasie hémorragique familiale (THF) est une maladie vasculaire rare héritée de manière autosomique dominante. Les patients atteints de THF peuvent présenter des dysplasies vasculaires nommées télangiectasies, ainsi que des malformations artérioveineuses. Notre objectif était de profiler et de caractériser des microARN (miRNA), courtes molécules d'ARN non codant régulatrices post-transcriptionnelles de l'expression génique, dans des cellules mononucléées de sang périphérique (CMSP) obtenues de patients atteints de THF. Les CMSP, comprenant majoritairement des lymphocytes et des monocytes, ont été décrits comme étant dysfonctionnels dans la THF. Au total, nous avons inscrit à l'étude 40 patients atteints de THF confirmée et 22 témoins. Nous avons isolé les CMSP à partir de 16 mL de sang périphérique et nous les avons purifiées pour obtenir l'ARN total. Le profil d'expression des miRNA a été obtenu à l'aide de l'analyse de puces (« arrays ») de miRNA humains. Les miRNA dysrégulés et les miRNA cibles sélectionnés étaient validés à l'aide de la technique de PCR quantitatif par transcriptase inverse. Des 377 miRNA criblés, nous avons identifié 41 miRNA dysrégulés. Nous avons observé que le miR-28-5p et le miR-361-3p, tous deux connus pour cibler l'IGF1 (pour « insulin-like growth factor 1 »), un puissant facteur de croissance angiogénique, subissaient une régulation à la baisse marquée chez les patients atteints de THF. Conséquemment, nous avons observé que les taux d'ARNm d'IGF1 étaient nettement plus élevés. Nos recherches ont permis d'établir avec succès la présence d'une dysrégulation des miRNA avec une augmentation des taux d'ARNm d'IGF1 dans les CMSP obtenues de patients atteints de THF. Cette nouvelle découverte représente peut-être un mode d'action pathogène que l'on pourrait cibler en vue d'atténuer les manifestations cliniques de la THF. [Traduit par la Rédaction]

Keywords

hereditary hemorrhagic telangiectasia; microRNA dysregulation; peripheral blood mononuclear cells

Mots-clés

télangiectasie hémorragique familiale; dysrégulation des microARN; cellules mononucléées de sang périphérique

Introduction

Hereditary hemorrhagic telangiectasia (HHT) is a rare, autosomal dominant, vascular disorder that affects 1 in 5000 to 10 000 people worldwide (Kjeldsen et al. 1999; Donaldson et al. 2014). HHT is characterized by angiogenic dysregulation leading to the formation of telangiectasias and arteriovenous malformations (AVMs). Telangiectasias, occurring in skin and mucocutaneous tissue (Shovlin et al. 2000; Faughnan et al. 2011), and organ AVMs are direct connections between arteries and veins without intervening capillary beds. Approximately 90% of HHT patients develop nasal telangiectasias that can cause chronic

epistaxis, and at least 20% develop chronic bleeding from gastrointestinal telangiectasias. (Shovlin et al. 2000; Faughnan et al. 2011). AVMs may develop in the pulmonary, hepatic, and cerebral vasculatures, which put the patient at risk of life-threatening hemorrhage and complications due to shunting, such as stroke, brain abscess, and high-output cardiac failure (Guttmacher et al. 1995; Shovlin et al. 2000; Faughnan et al. 2011).

HHT arises from heterozygous mutations in at least 3 known genes, endoglin (*ENG*, chromosomal locus 9q34), activin receptor-like kinase 1 (*ALK1*, also known as *ACVRL1*, chromosomal locus 12q1), and mothers against decapentaplegic homolog 4 (*SMAD4*, chromosomal locus 18q21) (McAllister et al. 1994; Johnson et al. 1996; Gallione et al. 2004). These genes encode proteins that are involved in the transforming growth factor beta (TGF- β)/bone morphogenetic protein (BMP) signalling pathway. *ENG* encodes for a TGF- β coreceptor, while *ACVRL1* encodes for a TGF- β type 1 receptor (Goumans et al. 2009; Pardali et al. 2010). *ENG* and *ACVRL1* mutations define HHT types 1 (HHT1) and 2 (HHT2), respectively, and generally display distinct clinical manifestations, although overlap of the clinical spectrum is not uncommon. *SMAD4* encodes for an intracellular TGF- β signalling protein, and mutations in *SMAD4* typically result in juvenile polyposis syndrome in addition to HHT (Johnson et al. 1996).

Haploinsufficiency of the respective gene products dysregulates TGF- β /BMP signalling, adversely affecting endothelial cell proliferation, migration, cell recruitment, and differentiation during angiogenesis (Bourdeau et al. 2000; Lebrin et al. 2004; Abdalla and Letarte 2006). Additionally, homozygous knockdown of *ENG* or *ACVRL1* in adult mice results in an HHT phenotype, confirming their role in HHT pathogenesis (Park et al. 2009; Choi et al. 2014; Garrido-Martin et al. 2014). Various cells in the inflammatory/repair response, including lymphocytes, monocytes/macrophages, neutrophils, and leukocytes, rely on TGF- β signalling, endoglin, and activin receptor-like kinase 1 for normal function (Shull et al. 1992; Kulkarni et al. 1993; Larsson et al. 2001; Ishida et al. 2004; Doetschman et al. 2012; Dingenouts et al. 2015). It has been hypothesized that AVM formation involves an impaired response to injury that may include the dysfunction of peripheral blood mononuclear cells (PBMCs) (Dingenouts et al. 2015).

PBMCs, which are composed mostly of lymphocytes and monocytes, have been implicated in HHT pathogenesis (Post et al. 2010; Venema 2015). In vivo HHT1 mouse models have demonstrated that aberrant TGF- β signalling impairs the homing capacity of PBMCs to sites of inflammation (Post et al. 2010). This reduced migratory capacity has been postulated to contribute to the development of angiogenic dysplasia, prolonged inflammation, and increased infection rates observed in HHT patients (Guilhem et al. 2013; Peter et al. 2014). Lymphopenia or reduced levels of CD4/CD8 T cells and natural killer cells have also been reported in HHT patients (Guilhem et al. 2013). However, the exact role PBMCs play in HHT pathogenesis is still unclear.

Micro-RNAs (miRNAs) are small (19 to 25 nucleotides long) endogenous noncoding RNA species that regulate gene expression posttranscriptionally through RNA interference (Lee et al. 1993; Wightman et al. 1993; Lagos-Quintana et al. 2001). These highly conserved species act as guides that deliver enzymatic complexes to silence mRNAs. MiRNAs have

been shown to silence mRNAs by 2 mechanisms that are dependent on miRNA–mRNA complementarity. Perfect complementarity leads to mRNA cleavage, whereas imperfect complementarity results in ribosome destabilization. Approximately 2000 miRNAs have been confidently identified in humans, and have been shown to be involved in a variety of human diseases, cellular functions, and pathways, including the TGF- β signalling pathway and angiogenesis (Wightman et al. 1993; Landgraf et al. 2007; Ardekani and Naeini 2010; Butz et al. 2012; Hammond 2015).

The expression and function of miRNAs associated with HHT remain largely unexplored. Our aim was to profile and characterize miRNAs in PBMCs from HHT patients through comparative miRNA array profiling and reverse transcription–quantitative polymerase chain reaction (RT–qPCR) validation. We hypothesize that the miRNA profile of PBMCs in HHT patients will be dysregulated compared to controls. We hope our findings will contribute to the understanding of the pathogenetic mechanisms involved in the development of HHT.

Materials and methods

Patient recruitment

A total of 16 mL of peripheral blood was obtained from patients clinically diagnosed with HHT according to Curaçao's diagnostic criteria for HHT (Shovlin et al. 2000) and from age- and gender-matched controls from the Toronto HHT Centre at St. Michael's Hospital, Toronto, Ontario, Canada. A total of 40 HHT patients between the ages 18 and 65 years were recruited. HHT patients who exhibited significant anemia (hemoglobin <100 g/L) or were pregnant were excluded from the study. Informed written consent was obtained from all participants involved in the study. All protocols that involved human samples were approved by the Research Ethics Board of St. Michael's Hospital, University of Toronto, in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Human peripheral blood mononuclear cell layer (buffy coat) isolation

Peripheral blood was collected into 2 BD Vacutainer CPT Mononuclear Cell Preparation Tubes and processed immediately. Blood samples were centrifuged at 4 °C for 30 min at 1650*g*. Half of the uppermost layer of plasma was aspirated and the buffy coat was collected. A total of 10 mL of cold (4 °C) PBS was added to the buffy coat and then it was centrifuged for 5 min at 300*g* to wash. The PBMC pellet was further processed for total RNA isolation.

Total RNA isolation of peripheral blood mononuclear cells

A Qiagen miRNeasy Mini Kit was used according to the manufacturer's protocol to isolate total RNA from PBMCs. The PBMC pellet was resuspended in 700 μ L of QIAzol lysis reagent and homogenized. The homogenate was incubated at room temperature (21–25 °C) for 5 min. Then 140 μ L of chloroform was added to the homogenate and it was vigorously shaken for 15 s. This mixture was incubated at room temperature (21–25 °C) for 3 min and then centrifuged at 4 °C for 15 min at 12 000*g*. The upper aqueous phase was transferred to a 1.5 mL Eppendorf tube, 1.5 volumes of 100% ethanol was added, and it was thoroughly

mixed. A maximum of 700 μL of this mixture was transferred to an RNeasy Mini column and centrifuged at 8000g for 15 s at room temperature (21–25 °C). The eluate was discarded. This procedure was repeated with the remainder of the sample. The column was washed with Buffer RWT and Buffer RPE in series via centrifugation. Finally, 40 μL of RNase-free water was added to the column and it was then centrifuged at 8000g for 1 min to elute the RNA. RNA quality and concentration was assessed with a NanoDrop 2000 spectrophotometer. RNA samples were stored at –80 °C until analysis. All equipment used was DNase-, RNase-, and pyrogen-free.

Micro-RNA array analysis of total RNA in peripheral blood mononuclear cells

An Applied Biosystems TaqMan Human MicroRNA Array covering 377 miRNAs (Card A v2.0) was used to profile the miRNAs in PBMCs. Prior to miRNA profiling, the samples were reverse transcribed into cDNA with a TaqMan MicroRNA Reverse Transcription Kit and Megaplex RT Primers. The RT reaction had a total volume of 7.5 μL , which included 3 μL of total RNA (600 ng), 0.8 μL of Megaplex RT primers (10X), 0.2 μL of dNTPs (100mmol/L), 1.5 μL of MultiScribe Reverse Transcriptase (50 U/ μL), 0.8 μL of 10X RT buffer, 0.9 μL of MgCl_2 (25 mmol/L), 0.1 μL of RNase inhibitor (20 U/ μL), and 0.2 μL of nuclease-free water. The thermocycling procedure was carried out as follows: (1) 40 cycles of 16 °C for 2 min, 42 °C for 1 min, and 50 °C for 1 s; (2) 85 °C for 5 min; and (3) 4 °C for ever. The PCR reaction had a total volume of 900 μL , which included 450 μL of 2X TaqMan Universal PCR Master Mix (no AmpErase UNG), 444 μL of nuclease-free water, and 6 μL of Megaplex RT product. Subsequently, 100 μL of the PCR reaction was dispensed into each port of the Array Card A v2.0. Following the manufacturer's instructions, the card was sealed and centrifuged. The PCR reaction was carried out with a 384-well TaqMan Low Density Array block on a ViiA 7 Real-Time PCR System (Applied Biosystems). PCR and thermocycling parameters were automatically inputted by the included SDS v2.2 setup file (SDS.txt). Results were analyzed using the RQ Study software (Applied Biosystems) and normalized to U6 RNA. MiRNAs of interest were selected based upon a fold change of 1.5 or greater and (or) consistency among samples (Liu et al. 2012; Sokolov et al. 2012).

Reverse transcription–quantitative polymerase chain reaction analysis for micro-RNAs

RT–qPCR was performed to validate select dysregulated miRNAs identified by the miRNA array analysis. Total RNA samples were reverse transcribed into cDNA with a TaqMan MicroRNA RT Kit (Applied Biosystems). Reaction components included 0.15 μL of dNTPs (100 mmol/L), 1 μL of MultiScribe Reverse Transcriptase (50 U/ μL), 1.5 μL of RT Buffer (10X), 0.19 μL of RNase inhibitor (20 U/ μL), 4.16 μL of nuclease-free water, 3 μL of RT primer (5X), and 5 μL of the RNA sample (total RNA 20 ng). The RT reaction was carried out as follows: 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, and 4 °C hold. MiRNA qPCR was done in a total volume of 10 μL with a Real-Time PCR Assay Kit (Applied Biosystems). The reaction contained 1 μL of RT product, 0.5 μL of PCR primer (20X), 5 μL of 2X TaqMan Universal PCR Master Mix (no AmpErase UNG), and 3.5 μL of nuclease-free water. The PCR reaction was carried out on a ViiA 7 Real-Time PCR System (Applied Biosystems) as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative quantification of individual miRNA expression was carried out with the 2^{-CT} method and normalized against endogenous U6 RNA.

Micro-RNA target identification

The characterization of miRNA function was accomplished using the published literature derived from the online journal databases Google Scholar and PubMed. MiRNA targets were identified and cross-referenced using the published literature and online miRNA databases, including miRTarBase (Chou et al. 2018), mirDIP (Tokar et al. 2018), mirPath v.3 (Vlachos et al. 2015), and TarBase v.8 (Karagkouni et al. 2018).

Reverse transcription–quantitative polymerase chain reaction analysis for mRNA

A total of 500 ng of purified RNA was used for RT with the Omniscript RT Kit (Qiagen). The total reaction volume was 20 μ L, which contained the following components: 2 μ L of 10X RT buffer, 1 μ L (10 U) of RNase inhibitor, 200 ng of random primer (Qiagen), 2 μ L of 5 mmol/L dNTPs, 1 μ L (4 U) of reverse transcriptase, and nuclease-free water (added to adjust the volume). The RT thermocycling protocol was as follows: 37 °C for 1 h followed by a 5 min incubation period at 85 °C to inactivate the enzyme. For *ENG* and *ACVRL1* mRNA expression, a SYBR Green gene assay kit (Thermo Fisher Scientific) was used. The sequences of the primers were as follows: *ENG*: 5'-AGCTGACTCTCCAGGCATCC-3' (forward), 5'-GCAGCTCTGTGGTGTGACC-3' (reverse); *ACVRL1*: 5'-GTGAGAGTGTGGCCGTCAG-3' (forward), 5'-CATGTCTGAGGCGATGAAGC-3' (reverse); and β -actin: 5'-AGCCTCGCCTTTGCCGA-3' (forward), 5'-CTGGTGCCTGGGGCG-3' (reverse). A total reaction volume of 10 μ L was used for qPCR that contained the following components: 5 μ L of 2X PowerUp SYBR Green Master Mix, 1 μ L of RT product, and 200 nmol/L each of primer and nuclease-free water (added to adjust the reaction volume). A ViiA7 qPCR System (Thermo Fisher Scientific) was used to perform qPCR. The thermocycling protocol used was 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A dissociation curve was generated for each reaction to determine the specificity of amplification. Normalization against β -actin was carried out to determine the relative levels of target genes with the 2^{-CT} method.

A TaqMan reporter probe was used to measure expression levels of IGF1 mRNA in PBMCs from HHT patients and controls. The primer sequence for the target was IGF1 forward 5'-GCTCACCTTACCAGCTCTGCCA-3' and IGF1 reverse 5'-TCCGACTGCTGGAGCCATACC-3'. The total volume of the qPCR reaction was 10 μ L, which contained the following components: 2 μ L RT product, 0.5 μ L TaqMan primer, 5 μ L of 2X TaqMan Universal PCR Master Mix (no AmpErase UNG), and 2.5 μ L of nuclease-free water. The qPCR thermocycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Normalization against β -actin was carried out to determine the relative levels of target genes with the 2^{-CT} method.

Enzyme-linked immunosorbent assay for IGF1 plasma protein

A human IGF1 enzyme-linked immunosorbent assay (ELISA) kit (Abcam, ab100545) was used to measure plasma IGF1 protein levels in HHT patients and controls. All reagents, standards, and samples were prepared according to the manufacturer's instructions. A total of 100 μ L of each plasma sample and standard were added to the appropriate wells and incubated with gentle shaking for 2.5 h at room temperature. After incubation, the solution was discarded, and the wells were thoroughly washed 4 times with 300 μ L of 1X Wash

Solution. After the final wash, residual liquid was removed and 100 μ L of 1X Biotinylated IGF1 Detection Antibody was added to each well and incubated for 1 h at room temperature with mild shaking. After incubation, the solution was discarded, and the wash step repeated. Then 100 μ L of 1X HRP-Streptavidin was added to each well and incubated with gentle shaking for 45 min at room temperature. The solution was discarded, the wash step was repeated, and 100 μ L of TMB One-Step Substrate Reagent was added to each well and incubated with gentle shaking for 30 min in the dark. Then 50 μ L of Stop solution was added to each well. The optical density at 450 nm was read using an eMax ELISA Microplate Reader. Data were analyzed by calculating the mean value of duplicated readings for each standard and sample.

Statistical analysis

A two-tailed Student's *t* test was used to determine significant differences between the means of the HHT patient and control groups. A *p* value of less than 0.05 was determined to be statistically significant.

Results

Participants

A total of 40 clinically confirmed HHT patients (27 females and 13 males) were enrolled in this study. The mean age of the HHT patient group was 45.2 ± 11.1 years and the mean age of the control group ($n = 22$; 15 females and 7 males) was 46.2 ± 12.9 years (Table 1). HHT patients with pulmonary AVMs were diagnosed by thoracic computed tomography (CT) or pulmonary angiography, patients with cerebral AVMs were diagnosed by magnetic resonance imaging (MRI), and patients with hepatic AVMs were diagnosed by contrast-enhanced CT, MRI, or Doppler ultrasonography. Mean age, gender, AVM type, and genotype of the HHT patient group as well as mean age and gender of the control group are shown in Table 1.

Decreased *ENG* and *ACVRL1* mRNA levels in HHT patient peripheral blood mononuclear cells

ENG and *ACVRL1* mRNA levels in the PBMCs of HHT patients and controls were relatively quantified by RT-qPCR and normalized using β -actin. *ENG* and *ACVRL1* mRNA levels were found to be significantly lower in HHT patient PBMCs compared to controls ($p < 0.01$ and < 0.05 , respectively) (Fig. 1). Relative levels of *ENG* mRNA in the HHT and control groups were 0.007 ± 0.0047 and 0.02 ± 0.013 , respectively; *ACVRL1* mRNA levels in the HHT patient and control groups were 0.0004 ± 0.0004 and 0.0015 ± 0.0010 , respectively. PBMCs displayed higher levels of *ENG* expression (combined mean C_T of HHT and control groups: 22.4) compared to *ACVRL1* (combined mean C_T of HHT and controls groups: 26.7) in both HHT patients and controls. In addition, *ENG* and *ACVRL1* mRNA levels were consistently decreased in both HHT1 ($n = 8$) and HHT2 ($n = 8$) patients.

Dysregulated micro-RNA array profile in HHT patient peripheral blood mononuclear cells

A total of 377 human miRNAs were analyzed in the array analysis. The analysis returned 41 dysregulated miRNAs, defined as having a fold change of 1.5 and greater, and 14 miRNAs

were selected based on consistency among samples. The dysregulated miRNAs included miRs-1-3p, -20b-5p, -28-5p, -29c-3p, -30b-5p, -133a3p, -143-3p, -214-3p, -361-3p, -374a-5p, -518d-3p, -618, -654-5p, and -708-5p (Fig. 2). Of these miRNAs, 9 were upregulated (miRs-1-3p, -20b-5p, -30b-5p, -133a-5p, -361-3p, -374a-5p, -618, -654-5p, and -708-5p) and 5 were downregulated (miRs-28-5p, -29c-3p, -143-3p, -214-3p, and -518d-3p). MiRs-28-5p, -30b-5p, -361-3p, and -374a-5p were selected for RT-qPCR validation because they were the most consistently dysregulated among the samples. Relative levels of all 14 dysregulated miRNAs are shown in Supplementary Fig. S1².

Decreased levels of miR-361-3p and miR-28-5p in HHT patient peripheral blood mononuclear cells

Level of miRs-28-5p, -30b-5p, -361-3p, and -374a-5p were validated by RT-qPCR using specific primers and U6 RNA as normalization in the PBMCs of HHT patients and controls. As shown in Figs. 3A and 3C, RT-qPCR validation returned significant changes of miR-28-5p and miR-361-3p levels between HHT patients and controls. Interestingly, miR-361-3p was determined to be upregulated by the array analysis but downregulated as determined by RT-qPCR. It is well known that variability exists between arrays and RT-qPCR, but RT-qPCR remains the gold standard for the relative quantification of miRNAs and is necessary to validate miRNA array data (Git et al. 2010; Chugh and Dittmer 2012). MiR30b-5p and miR-374a-5p levels were not found to be significantly different between HHT patients and controls (Figs. 3B and 3D, respectively). Relative levels of miR-30b-5p were 0.146 ± 0.021 and 0.164 ± 0.019 for HHT patient and control groups, respectively. MiR-374a-5p demonstrated relative levels of 0.067 ± 0.026 and 0.079 ± 0.008 for HHT patient and control groups, respectively. However, miR-28-5p and miR-361-3p were found to be significantly decreased in HHT patient PBMCs compared to controls ($p < 0.05$) (Figs. 3A and 3C, respectively). Relative levels of miR-28-5p were 0.009 ± 0.001 and 0.013 ± 0.001 for HHT patient and control groups, respectively. MiR-361-3p returned relative levels of 0.002 ± 0.001 and 0.004 ± 0.001 for HHT patient and control groups, respectively. MiR-28-5p and miR-361-3p were shown to both target IGF1 based on published literature and various miRNA data-bases: miRTarBase (Chou et al. 2018), mirDIP (Tokar et al. 2018), mirPath v.3 (Vlachos et al. 2015), and TarBase v.8 (Karagkouni et al. 2018).

Increased IGF1 micro-RNA levels in HHT patient peripheral blood mononuclear cells

IGF1 mRNA levels were quantified by RT-qPCR and normalized to β -actin. Levels of IGF1 mRNA were significantly increased in HHT patients compared to controls ($p < 0.01$), shown in Fig. 4. Relative levels of IGF1 mRNA in HHT patients and controls were $2.1\text{E}-5 \pm 1.7\text{E}-5$ and $6.09\text{E}-6 \pm 4.2\text{E}-6$, respectively. PBMCs from HHT patients consistently displayed higher expression of IGF1 (mean C_T : 29) compared to control PBMCs (mean C_T : 31). Additionally, IGF1 plasma protein levels were not significantly different between HHT patient and control groups. Plasma IGF1 protein levels in HHT patients and controls were 95 ± 21 ng/mL and 105 ± 16 ng/mL, respectively (Fig. 5).

²Supplementary data are available with the article through the journal Web site at <http://nrcresearchpress.com/doi/suppl/10.1139/cjpp-2018-0508>.

Discussion

HHT is a rare genetic disorder inherited in an autosomal dominant fashion in which patients develop hemorrhagic vascular dysplasias called telangiectasias and AVMs. PBMCs are a heterogeneous cellular population, comprised of lymphocytes and monocytes, which provide an in vitro model for studying processes such as immunity, inflammation, vascular repair, and angiogenesis (Dingenouts et al. 2015). PBMC dysfunction has been identified in HHT in the form of lymphopenia and reduced monocyte migration (Post et al. 2010; Guilhem et al. 2013); however, the exact role of PBMCs in HHT pathogenesis is still unclear. In addition, how miRNAs might be involved in HHT is not fully understood. Our goal was to profile and characterize miRNAs in HHT patient-derived PBMCs.

Sanz-Rodriguez et al. (2004) have identified reduced *ENG* up-regulation in activated monocytes from HHT1 and HHT2 patients. In the present study, we determined that freshly isolated PBMCs from both HHT1 and HHT2 patients displayed significantly reduced *ENG* and *ACVRL1* mRNA levels. Of interest, decreased *ENG* and *ACVRL1* expression was exhibited in both HHT1 and HHT2 patients. This suggests a reciprocal relationship between the expression of *ENG* and *ACVRL1*.

Research on miRNA dysregulation in HHT is quite limited. We have previously identified elevated miR-210 levels in the circulation of HHT patients with pulmonary AVMs (Zhang et al. 2013) and have suggested that miR-210 may represent a valuable biomarker for the identification of pulmonary AVMs in patients with HHT. A study by Tabruyn et al. (2013) that same year reported that miR205, an inhibitor of TGF- β signalling, was downregulated in the circulation of HHT1 and HHT2 patients. In this study, we identified miRNA dysregulation in PBMCs derived from HHT patients that included a significant downregulation of miR-28-5p and miR-361-3p shown by RT-qPCR. MiRs-143-3p, -518d-3p, -618, and -654-5p demonstrated the largest fold changes, but were not found to be enriched in HHT patient and control PBMCs. Additionally, these miRNAs are not well characterized or known to play a functional role in PBMCs. MiR-361-3p has been shown to function in potentially proangiogenic Tie2-expressing monocytes through the regulation of IGF1 (Wang et al. 2016). MiR-28-5p has also been shown to target IGF1, with the overexpression of miR-28-5p resulting in a significant reduction of IGF1 transcripts in hepatocellular carcinoma cells (Shi and Teng 2015). Here we show that IGF1 mRNA levels are significantly increased in PBMCs from HHT patients compared to controls. Therefore, it is possible that the increase in IGF1 transcripts is a result of the decreased levels of miR-28-5p and miR-361-3p. PBMCs were studied as a whole because of the importance of monocyte-lymphocyte interaction in the angiogenic process (Naldini et al. 2003; Naldini and Carraro 2005; Ramello et al. 2014). The study of individual cells from this population could limit the importance of this interaction.

PBMCs, specifically the monocyte fraction, have been shown to express and secrete IGF1 in a paracrine manner to modulate and coordinate various processes, including inflammation, vascular repair, and angiogenesis (Delafontaine et al. 2004; Dimova and Djonov 2017). Additionally, PBMCs have been reported to express the IGF1 receptor and are susceptible to IGF1 stimulation (Kooijman et al. 1992). Recently, the phosphatidylinositol 3-kinase/

protein kinase B (PI3K/AKT) pathway has been implicated in HHT pathogenesis, where its inhibition in an HHT2 mouse model attenuated AVM formation (Ola et al. 2016). It has been shown that IGF1 plays a predominant role in the regulation of angiogenesis through the stimulation of the PI3K/AKT pathway (Delafontaine et al. 2004; Bach 2015; Friedrich et al. 2018). Our finding of similar IGF1 plasma protein levels between HHT patients and controls suggests that IGF1 may be overexpressed locally at sites of inflammation or wounding, thereby stimulating abnormal angiogenesis and potentially contributing to AVM formation without necessarily increasing systemically measured IGF1. This postulation is in line with the 3-event hypothesis that describes the requirement of a local angiogenic trigger (i.e., inflammation, infection or wounding) for AVM formation to occur (Tual-Chalot et al. 2015). PBMCs and IGF1 could be involved in these processes and play an important role in HHT pathogenesis.

In conclusion, we identified miRNA dysregulation in PBMCs derived from HHT patients. The findings of decreased miR-28–5p and miR-361–3p levels and elevated IGF1 mRNA levels may represent an important pathogenic mechanism that warrants further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Reverse transcription–quantitative polymerase chain reaction (RT–qPCR) of *ENG* and *ACVRL1* mRNA levels in hereditary hemorrhagic telangiectasia (HHT) patient ($n = 8$) and control ($n = 6$) peripheral blood mononuclear cells. (A) RT–qPCR of *ENG* mRNA levels in HHT patients compared to controls. (B) RT–qPCR *ACVRL1* mRNA levels in HHT patients compared to controls. * $p < 0.05$; ** $p < 0.01$.

Fig. 2.

Fold change of dysregulated micro-RNAs (MiRNAs) in hereditary hemorrhagic telangiectasia (HHT) patient peripheral blood mononuclear cells (PBMCs) relative to controls. Of the 377 miRNAs screened, 41 miRNAs were dysregulated, defined as a fold change of 1.5 or greater, and 14 miRNAs were selected based on consistency of dysregulation in HHT patients and controls. Of the 14 miRNAs, 9 were upregulated and 5 were downregulated. MiR-654-5p returned the largest positive fold change of 5, whereas miR-361-3p demonstrated the lowest positive fold change of 1.5. MiR-143-3p demonstrated the largest negative fold change of 3. MiR-28-5p returned a negative fold change of 1.8, miR-30b-5p returned a positive fold change of 1.5, and miR-374a-5p demonstrated a positive fold change of 1.6.

Fig. 3. Reverse transcription–quantitative polymerase chain reaction (RT–qPCR) of miRs-28–5p, –30b–5p, –361–3p, and –374a–5p levels in hereditary hemorrhagic telangiectasia (HHT) patient ($n = 5$) and control ($n = 5$) peripheral blood mononuclear cells. (A) RT–qPCR of miRNA-285p in HHT patients compared to controls. (B) MiRNA-30b-5p levels in HHT patients compared to controls. (C) RT–qPCR of miRNA-361–3p in HHT patients compared to controls. (D) MiRNA-374a-5p levels in HHT patients compared to controls. * $p < 0.05$.

Fig. 4. Reverse transcription–quantitative polymerase chain reaction of IGF1 mRNA levels in hereditary hemorrhagic telangiectasia (HHT) patient ($n = 14$) and control ($n = 13$) peripheral blood mononuclear cells. ** $p < 0.01$.

Fig. 5. ELISA of IGF1 protein levels in hereditary hemorrhagic telangiectasia (HHT) patient ($n = 24$) and control ($n = 16$) plasma. The mean amount of IGF1 protein in the HHT patient group and control group was 95 ± 21 ng/mL and 105 ± 15 ng/mL, respectively.

Table 1.

Summary of demographics of patients with hereditary hemorrhagic telangiectasia (HHT) and controls.

	HHT Patients (n = 40)	Controls (n = 22)
Age (years), mean ± SD	45.2±11.1	46.2±12.9
Females, n (%)	27 (67.5)	15 (68.2)
Female age (years), mean ± SD	43.2±10.4	46.5±11.7
Males, n (%)	13 (32.5)	7 (31.8)
Male age (years), mean ± SD	49.6±11.7	45.5±16.1
Mutations, n (%)		
<i>ENG</i>	19 (47.5)	
<i>ACVRL1</i>	14 (35)	
<i>SMAD4</i>	0 (0)	
Unknown ^a	2 (5)	
Undetermined ^b	5 (12.5)	
Patients with AVMs, n (%)		
PAVM	23 (57.5)	
PAVM and CAVM	2 (5)	
PAVM and LVM	4 (10)	
CAVM	4 (10)	
No AVM	7 (17.5)	

Note: *ACVRL1*, activin receptor-like kinase 1; AVM, arteriovenous malformation; CAVM, cerebral arteriovenous malformation; *ENG*, endoglin; LVM, liver arteriovenous malformation; PAVM, pulmonary arteriovenous malformation; *SMAD4*, mothers against decapentaplegic homolog 4.

^aPatients screened negative for known HHT mutations, including *ENG*, *ACVRL1*, and *SMAD4*.

^bPatients that had not yet undergone genetic testing.