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PRELIMINARY COMMUNICATIONS

Elevated circulating microRNA-210 levels in patients with hereditary hemorrhagic telangiectasia and pulmonary arteriovenous malformations: a potential new biomarker

Qiuwang Zhang[1], Ivana Kandic[1], Marie E. Faughnan[2], and Michael J. Kutryk[3]

[1]Division of Cardiology, Keenan Research Center at the Li Ka Shing Knowledge Institute, St. Michael's Hospital, University of Toronto, Toronto, Ontario, Canada, [2]Division of Respirology, Keenan Research Center at the Li Ka Shing Knowledge Institute, St. Michael's Hospital, University of Toronto, Toronto, Ontario, Canada, and [3]Division of Cardiology, St. Michael's Hospital, University of Toronto, Toronto, Ontario, Canada

Abstract

Pulmonary arteriovenous malformations (PAVMs), which can lead to life-threatening bleeding and other complications, have been reported to occur in 30–50% of patients with hereditary hemorrhagic telangiectasia (HHT). Circulating microRNAs (miRNAs) have emerged as new biomarkers for human diseases. This study was conducted to explore circulating miRNAs as biomarkers for the screening of HHT patients with PAVMs. MicroRNA array analysis revealed eight altered circulating miRNAs in patients with PAVMs. Real time RT-PCR showed that the levels of circulating miR-210 were significantly elevated in HHT patients with PAVMs but not changed in patients without PAVMs as compared with healthy controls. Circulating miR-210 therefore may be used as a new and sensitive biomarker for the screening of patients with HHT for clinically significant PAVMs.

Keywords: hereditary hemorrhagic telangiectasia, pulmonary arteriovenous malformation, microRNA, biomarker, real time RT-PCR

Introduction

Hereditary hemorrhagic telangiectasia (HHT), also known as Osler-Weber-Rendu syndrome, is an autosomal dominant genetic disease that affects approximately 1 in 5-8000 people (Plauchu et al., 1989; Govani & Shovlin, 2009). HHT is a vascular disorder with a variety of clinical manifestations. Patients with HHT often show characteristic mucocutaneous telangiectasia, and common clinical presentations are epistaxis, gastrointestinal bleeding, and iron deficiency anemia. Arteriovenous malformations (AVMs) can occur in the pulmonary (PAVMs), hepatic, and cerebral circulations, and PAVMs have been reported to occur in 30–50% of HHT patients (Guttmacher et al., 1995; Shovlin et al., 2000; Shovlin, 2010). Irrespective of their location or size, the abnormal blood vessels in HHT have a greater tendency to rupture and bleed. Screening patients with HHT is crucial in order to identify those patients who might be at risk for AVM rupture and other complications, and to determine who would benefit from treatment of those AVMs, i.e. PAVMs and brain AVMs, which might be life threatening.

Since HHT is characterized by genetic heterogeneity, most HHT patients are usually defined as affected by HHT type-1 or type-2, according to which gene carries the disease-causing mutation, although the two types show a considerably overlapping clinical spectrum. HHT1 is caused by mutation in the endoglin gene (chromosomal locus 9q34) that encodes endoglin (*ENG*), while HHT2 is caused by mutation in the ALK1 (*ACVRL1*) (chromosomal locus 12q1) that encodes activin receptor-like kinase 1 (ALK1) (McAllister et al., 1994; Johnson et al., 1996). Both proteins participate in the signal cascade of transforming growth factor-β/bone morphogenetic protein (TGFβ/BMP) superfamily, an important pathway modulating

Address for Correspondence: Dr. Michael J. Kutryk, St. Michael's Hospital, University of Toronto, Room 7084, Bond Wing, 30 Bond Street, Toronto, Ontario, Canada M5B 1W8. Tel. (416)-864-6060 ext. 6155. Fax (416)-864-5989. E-mail: kutrykm@smh.ca

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several cellular processes, including the regulation of angiogenesis. Aberrant endoglin or ALK1 signaling results in excessive endothelial cell proliferation and impaired recruitment of mural cells during angiogenesis, leading to abnormal blood vessel formation (Lebrin et al., 2004; Park et al., 2009). In vivo studies of animal models have shown that heterozygous mice carrying only one copy of the respective gene (i.e. *eng*+/– or *acvrl*1+/–) display features of HHT, establishing a causal role of the genes in the pathogenesis of HHT (Bourdeau et al., 1999; Srinivasan et al., 2003).

MicroRNAs (miRNAs) are a class of endogenous, highly conserved noncoding RNAs of approximately 22 nucleotides in length, which negatively regulate gene expression post-transcriptionally (Lee et al., 1993; Wightman et al., 1993). They regulate over 30% of genes in a cell via degradation or translational inhibition of their target mRNAs. First described in 1993, miRNAs have been recognized as important regulators of a wide range of cellular processes (Ambros, 2004; Bartel, 2004). Recently, circulating miRNAs have emerged as new and sensitive biomarkers for the diagnosis, progression or prognostication of a variety of diseases, including cardiovascular disease, and some cancers and infectious diseases (Mitchell et al., 2008; Fichtlscherer et al., 2010; Zampetaki et al., 2010; D'Alessandra et al., 2010; Ji et al., 2011).

In this study, we recruited HHT patients without PAVMs as well as patients with documented PAVMs. Comparative miRNA array profiling of plasma samples was performed and real time RT-PCR was used to validate the array data. Our goal was to determine whether circulating miRNAs can serve as biomarkers for the screening of HHT patients with PAVMs.

Methods

Recruitment of patients

Patients clinically diagnosed with HHT and age- and gender-matched healthy volunteers were recruited. All patients with HHT included in this study underwent chest computed tomography (CT) scans to identify those with PAVMs, and many with PAVMs underwent confirmatory pulmonary angiography. Written informed consent for study involvement was obtained from all participants. All protocols involving human samples were approved by the Research Ethnics Board of St. Michael's Hospital, University of Toronto, in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

Plasma preparation

Peripheral blood was collected into K2-EDTA tubes (Beckton Dickinson, BD) and processed within 5 min for plasma preparation. Blood samples were first centrifuged at 1500 g for 15 min at 4°C. The supernatant was collected and transferred to nuclease-free tubes and centrifuged again at 14000 g for 15 min at 4°C. The supernatant was processed further for total RNA extraction or aliquoted and stored at –80°C.

Isolation of total RNA from human plasma

A miRNeasy Mini Kit (Qiagen) was used to isolate total RNA from human plasma according to the manufacturer's instructions with cel-miR-39 (Qiagen) spiked for normalization of the RNA preparation. Briefly, 200 µl of plasma was mixed with 1000 µl QIAzol lysis reagent and placed at room temperature for 5 min. Two µl of 0.5 nM cel-miR-39 was added to the homogenate followed by extraction with 200 µl of chloroform. After centrifugation at 12000 rpm for 15 min, the upper aqueous phase containing RNA was transferred into a nuclease free tube and 1.5 volumes of 100% ethanol was added. The sample was loaded into an RNeasy Mini spin column which was placed in a 2 ml collection tube. After a brief centrifugation, the column was washed with buffers RWT and RPE sequentially with the aid of centrifugation. RNA was eluted finally with 40 µl nuclease-free water.

MicroRNA array

The TaqMan human microRNA array A (Card A) version 2.0 (Applied Biosystems Inc.) was used for miRNA expression profiling of the plasma sample. Before array profiling, total RNA was reverse transcribed (RT) and RT reaction product was further pre-amplified. The Human Megaplex Primer Pool A version 2.0 (Applied Biosystems Inc.) was used for RT reaction which was carried out in a total volume of 7.5 µl containing: 3 µl of total RNA, 0.8 µl of human pool A megaplex primers, 0.2 µl of 100 mM dNTPs, 0.8 µl of 10x reverse-transcription buffer, 0.9 µl of 25 mM MgCl2, 1.5 µl of multiscribe reverse-transcriptase (50U/ µl), 0.1 µl of RNase inhibitor (20U/µl) and 0.2 µl of nuclease-free water. The RT reaction was done in 40 cycles of 16°C for 2 min, 42°C for 1 min, and 50°C for 1 s. For pre-amplification, the Megaplex PreAmp Primers A version 2.0 (Applied Biosystems Inc.) was used and the reaction contained following components: 2.5 µl of the RT product, 12.5 µl of 2x preamplification master mix, 2.5 µl of 10x Megaplex PreAmp primers, and 7.5 µl of nuclease-free water. The pre-amplification reaction was performed by heating the samples at 95°C for 10 min, followed by 12 cycles of 95°C for 15 s and 60°C for 4 min. The pre-amplified product was diluted to a final volume of 100 µl with nuclease free water. For miRNA array assay, 10 µl of the diluted pre-amplification product was mixed with 450 µl of 2x Taqman Universal PCR Master Mix and 440 µl nuclease free water. After mix, 100 µl of the mixture was dispensed to each port of the array card A. The card was centrifuged and sealed according to the manufacturer's instructions. The reaction was then performed on an Applied Biosystems 7900HT thermocycler using the SDS2.3 Software (Applied Biosystems Inc.) and analysis of the results was performed using the RQ analysis program (Applied Biosystems Inc.).

Table 1 Genotypes and the Curacao criteria present in the patients.

Patient	PAVM	Mutation identity	Number of Curacao criteria present
1	+	Not genotyped	3
2	–	Not genotyped	3
3	+	No mutations identified	3
4	+	Alk 1 mutation	3
5	+	Endoglin mutation	4
6	+	Endoglin mutation	3
7	+	Alk mutation	2
8	–	Not genotyped	3
9	–	Not genotyped	3
10	–	Not genotyped	3
11	–	Not genotyped	3
12	+	Not genotyped	3
13	–	No mutations identified	3
14	+	No mutations identified	3
15	–	Not genotyped	3

PAVM, pulmonary arteriovenous malformations.

Quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) analysis

Quantitative RT-PCR was carried out to detect selected miRNAs to verify the array data. A TaqMan miRNA reverse transcription kit (Applied Biosystems Inc.) was used for first-strand cDNA synthesis. The reverse transcription reaction contained following components: 0.2 µl 100 mM dNTP, 1.5 µl 10X RT buffer, 3 µl individual miRNA specific primers, 1 µl multiscribe reverse transcriptase (50 U/ µl), 3 µl RNA, and 6.3 µl of nuclease free water. RT was accomplished in following steps: 16°C for 30 min; 42°C for 30 min; 85°C for 5 min. Real time PCR was performed by using the Real Time PCR Assay Kit (Applied Biosystems Inc.). The reaction had a total volume of 20 µl containing: 2 µl RT product, 1 µl 20x TaqMan microRNA assay primer, 10 µl 2xTaqMan universal PCR master mix and 7 µl of nuclease-free water. Real time PCR was run on the 7900HT DNA sequence detection system as follows: stage 1, 95°C for 10 min, stage 2, 95°C for 15 s, and 60°C for 1 min. Stage 2 was repeated for 40 cycles. The relative expression level of each individual miRNA after normalization to the spiked cel-miR-39 was calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical significance was determined by using Student's t test; $p < 0.05$ was considered statistically significant.

Results

Study subjects

Two patient groups, one with PAVMs (n = 8) and the other without PAVMs (n = 7) as determined by chest CT scan and/or pulmonary angiogram, were included in this study. The average age for the PAVM patient group was 54.9 ± 16.8 years (range 38–68), and patients without PAVMs had an average age of 52.4 ± 15.4 years (range 23–63). The average age of the healthy controls (n = 7) was 52.5 ± 9.6 years (range 34–60). The genotype of each patient and the Curacao criteria defining the HHT condition were shown in Table 1.

Table 2 MicroRNAs detected in the plasma samples by array analysis.

hsa-let-7a	hsa-miR-106a	has-miR-222
hsa-let-7b	hsa-miR-106b	has-miR-223
hsa-let-7c	hsa-miR-122	has-miR-301a
hsa-let-7d	hsa-miR-125b	has-miR-320
hsa-let-7e	hsa-miR-126	hsa-miR-323-3p
hsa-let-7f	hsa-miR-128	hsa-miR-324-3p
hsa-let-7g	hsa-miR-130a	hsa-miR-324-5p
hsa-miR-10a	hsa-miR-130b	hsa-miR-328
hsa-miR-10b	hsa-miR-132	hsa-miR-331-3p
hsa-miR-15b	hsa-miR-133a	hsa-miR-335
hsa-miR-16	hsa-miR-138	hsa-miR-340
hsa-miR-17	hsa-miR-139-5p	has-miR-155
hsa-miR-18a	hsa-miR-140-3p	hsa-miR-342-3p
hsa-miR-18b	hsa-miR-140-5p	hsa-miR-345
hsa-miR-19a	hsa-miR-142-3p	hsa-miR-365
hsa-miR-19b	hsa-miR-142-5p	hsa-miR-374a
hsa-miR-20a	hsa-miR-143	hsa-miR-374b
hsa-miR-20b	hsa-miR-145	hsa-miR-375
hsa-miR-21	hsa-miR-146a	hsa-miR-423-5p
hsa-miR-22	hsa-miR-146b-5p	hsa-miR-425
hsa-miR-24	hsa-miR-148a	hsa-miR-451
hsa-miR-25	hsa-miR-148b	hsa-miR-454
hsa-miR-26a	hsa-miR-150	hsa-miR-483-5p
hsa-miR-26b	hsa-miR-152	hsa-miR-484
hsa-miR-27a	hsa-miR-181a	hsa-miR-486-3p
hsa-miR-28-3p	hsa-miR-185	hsa-miR-486-5p
hsa-miR-29a	hsa-miR-186	hsa-miR-505
hsa-miR-29c	hsa-miR-192	hsa-miR-518f
hsa-miR-30c	hsa-miR-193a-5p	hsa-miR-532-5p
hsa-miR-34a	hsa-miR-197	hsa-miR-574-3p
hsa-miR-92a	hsa-miR-199a-3p	hsa-miR-590-5p
hsa-miR-93	hsa-miR-210	hsa-miR-598
hsa-miR-99a	hsa-miR-214	hsa-miR-652
hsa-miR-100	hsa-miR-215	hsa-miR-660
hsa-miR-101	hsa-miR-218	hsa-miR-885-5p
hsa-miR-103	hsa-miR-221	hsa-miR-376c

Circulating miRNA array profiling

Human microRNA array card A (Applied Biosystems Inc.), covering 377 miRNAs, was used to comparatively profile miRNA expression in plasma samples of study subjects. As shown in Table 2, a total of 108 miRNAs were detected from all six samples (three PAVM patients and three healthy volunteers) as assessed by the Ct value (<37) as well as the amplification curve. Of these detectable miRNAs, eight were found consistently either up- or down-regulated over three fold in patients compared with healthy subjects (miR-7e, miR-10a,

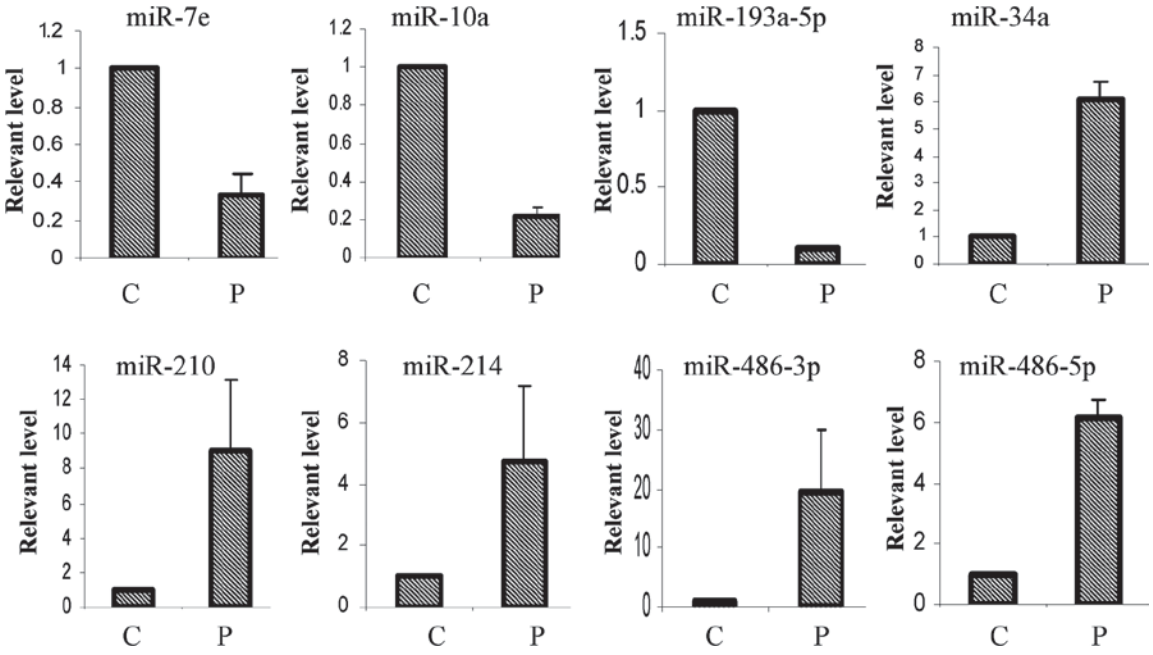

Figure 1. Circulating miRNA level changes in HHT patients detected by miRNA array analysis. Array profiling of circulating miRNAs showed that levels of circulating miR-7e, miR-10a and miR-193a-5p were reduced while levels of miR-34a, miR-210, miR-214, miR-486-3p and miR-486-5p were elevated in all patients with PAVM (n=3) compared with healthy controls (n = 3). For comparison, the level of each miRNA from health controls was set as 1. C = healthy control, P = patients with PAVM. HHT, hereditary hemorrhagic telangiectasia; PAVM, pulmonary arteriovenous malformations.

mir-193a-5p were decreased while miR-34a, miR-210, miR-486-3p and miR-486-5p were increased, Figure 1). Although many targets for miR-7e, miR-193a-5p, miR-486-3p and miR-486-5p have been predicted based on sequence homology (www.mirbase.org), the precise functions of these miRNAs remain unknown. MiR-10a and miR-34a have been shown to be involved in endothelial inflammation and senescence (Fang et al., 2010; Ito et al., 2010). MiR-210 is regulated by hypoxia (Kulshreshtha et al., 2007). These three miRNAs therefore were chosen for further analysis with real time RT-PCR.

Validation of the spiked cel-miR-39 as an internal control

As there are no known miRNAs which can serve as internal controls for human plasma and serum samples, an external miRNA is commonly used for normalization (Mitchell et al., 2008; Fichtlscherer et al., 2010). In the present study, a *Caenorhabditis elegans* miRNA, i.e. cel-miR-39, was added to the plasma during RNA preparation procedure as described above. Two HHT and 2 healthy control samples were randomly chosen for detection of the levels of the spiked cel-miR-39 by real time RT-PCR. As shown in Figure 2, the Ct values of cel-miR-39 from those 4 samples were consistent, ranging from 23.83 to 24.02, validating the spiked foreign C. elegans miRNA as a control. Thereby, the level of each individual miRNA detected by real time RT-PCR was normalized against the cel-miR-39 level.

Elevated circulating miR-210 level in HHT patients with PAVM

Circulating miR-10a, miR-34a and miR-210 were relatively quantified first in patients with PAVM and healthy controls by real time RT-PCR using each individual miRNA specific primers, and the results were normalized against the spiked cel-miR-39. As shown in Figure 3, changes of all three miRNAs in patients with PAVMs appeared to be in line with the array data. Levels of miR-34a were 0.0356 ± 0.0187 and 0.0189 ± 0.0037 ($p = 0.40$) for PAVM patients and healthy subjects, respectively, and levels of miR-10a were 0.0133 ± 0.0028 and 0.0242 ± 0.0085 ($p = 0.25$) for PAVM patients and healthy subjects, respectively. However, changes of these two miRNAs were not statistically significant. In contrast, miR-210, a hypoxia-inducible miRNA was significantly, and consistently, increased in HHT patients with PAVMs (0.0372 ± 0.0068) compared with healthy controls (0.0150 ± 0.0032, $p < 0.05$). Furthermore, a group of HHT patients without PAVMs did not show a change in plasma miR-210 levels (0.0163 ± 0.0038, $p = 0.86$, compared with healthy subjects).

Discussion

It has been well established that miRNAs are involved in the regulation of many aspects of the cardiovascular system (Poliseno et al., 2006; Wang et al., 2008; Bonauer et al., 2009; Fish et al., 2011). There is also mounting evidence that circulating miRNAs may serve as sensitive biomarkers for cardiovascular diseases. Fichtlscherer

et al. studied circulating miRNAs in patients with stable coronary artery disease and found that endothelial specific miRNAs, e.g. miR-126 and miR-92a were decreased in patients. In contrast, muscle enriched miRNAs, e.g. miR-133a and miR-208a were increased (Fichtlscherer et al., 2010). Zampetaki and colleagues reported the lower plasma levels of miR-20b, miR-21, miR-24, miR-15a, miR-126, miR-191, miR-197, miR-223, miR-320, and miR-486 in patients with type 2 diabetes (Zampetaki et al., 2010). Circulating miRNAs have also been examined in patients suffering acute myocardial infarction, and the results show modulation of miRNA plasma levels both in humans as well as in a mouse model of myocardial infarction (D'Alessandra et al., 2010).

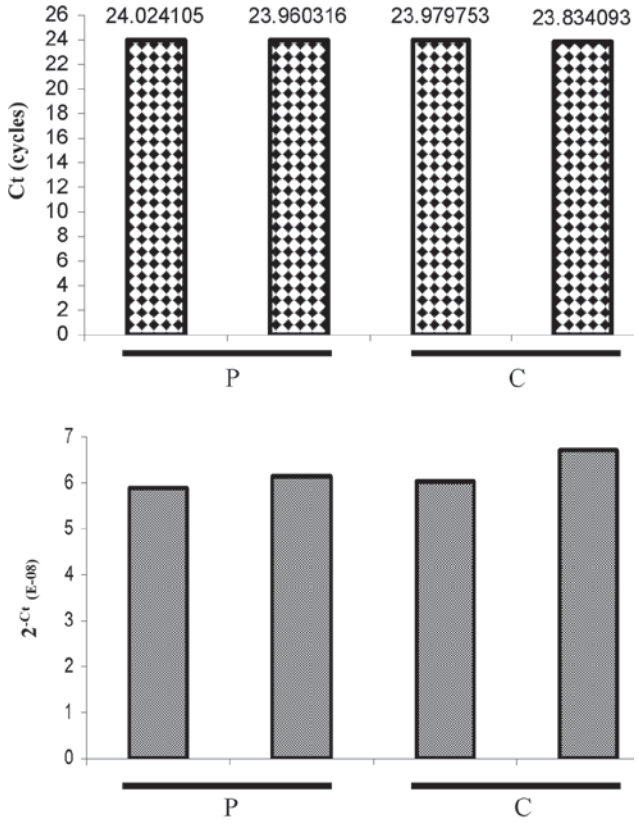

Figure 2. Validation of the spiked cel-miR-39 as an internal control. Spiked cel-miR-39 was detected in four randomly selected samples (two healthy controls and two patients with PAVM), and the Ct values were shown on the top of each bar in the top panel. Bottom panel showed the amount of cel-miR-39 in each sample as calculated by 2^{-Ct}. P = patients with PAVM, C = healthy control. PAVM, pulmonary arteriovenous malformations.

HHT is a genetic disease that affects the vascular system and can result in a variety of clinical manifestations (Guttmacher et al., 1995; Shovlin, 2010). PAVMs are common in patients with HHT, and have been associated with life-threatening complications such as stroke, transient ischemic attack, cerebral abscess, massive hemoptysis, and spontaneous hemothorax (Faughnan et al., 2011). Since clinical symptoms and signs of PAVMs are often absent before the development of complications, current guidelines recommend that all patients with HHT be screened for the presence of PAVMs at the time of their initial clinical evaluation, and repeated every 5–10 years (Faughnan et al., 2011). Screening can be performed using physiologic methods as well as various sophisticated imaging modalities.

We hypothesized that abnormal blood vessel formation in HHT results in dysregulation of miRNAs, which will manifest in circulating miRNA levels as seen in other cardiovascular diseases. In the present study, we identified eight miRNAs whose circulating levels changed in HHT patients with PAVMs by microRNA array technology. Using real time RT-PCR analysis, we reported here that circulating miR-210 levels were significantly elevated in HHT patients with PAVMs but not changed in patients without PAVMs. As excessive endothelial cell proliferation and a paucity of vascular smooth muscle mural cells, in small vessels are typical phenotypic findings in patients with HHT, we speculated that circulating miRNAs with vascular targets would be affected. Unexpectedly, the levels of miRNAs thought to be endothelial (miR-216, miR-92a and miR-221/222), or vascular smooth muscle cell (miR-143 and miR-145) selective were not altered in patients (data not shown).

MiR-210 is a unique hypoxamir that is ubiquitously induced in both transformed and primary cell types (Hua et al., 2006; Camps et al., 2008; Fasanaro et al., 2008;

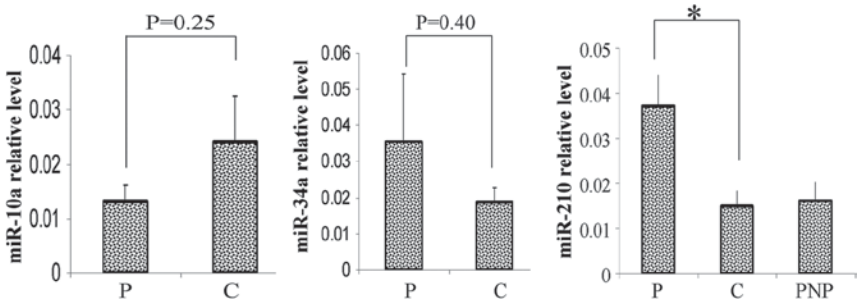

Figure 3. Real time RT-PCR analysis revealed the elevation of circulating miR-210 in HHT patients with PAVM but not in patients without PAVM. MiRNAs of interest (miR-10a, miR-34a and miR-210) were further analyzed by real time RT-PCR. The result showed that circulating miR-210 level in PAVM patients (0.0372 ± 0.0068) was significantly increased while not changed in patients without PAVM (0.0163 ± 0.038) compared with healthy controls (0.0150 ± 0.0032). *$p < 0.05$. C = healthy control, P = patients with PAVM, PNP = patients with no PAVM. HHT, hereditary hemorrhagic telangiectasia; PAVM, pulmonary arteriovenous malformations.

Giannakakis et al., 2008; Pulkkinen et al., 2008; Crosby et al., 2009; Zhang et al., 2009; Chan & Loscalzo, 2010). To date, at least 37 unique transcripts have been identified and further verified as direct targets of repression by miR-210 (Chan & Loscalzo, 2010; Kelly et al., 2011; Cui et al., 2012; Qi et al., 2012). These transcripts are associated with a myriad of cellular functions that are critical for adaptation to hypoxic stress such as RNA and DNA processing, binding and repair; cell survival, migration, adhesion and differentiation; cell cycle control; cytoplasmic and nuclear trafficking; mitochondrial metabolism; protein modification; and angiogenesis. Numerous studies have revealed high expression of miR-210 in tumor tissues, including breast, ovarian, and head and neck cancers and miR-210 levels are inversely correlated with overall survival in cancer patients, making it a potential biomarker for prognostication of various cancers (Camps et al., 2008; Gee et al., 2010; Greither et al., 2012; Rothé et al., 2011). In a study examining the miRNA signature of hypoxia, Kulshreshtha and colleagues identified a specific spectrum of miRNAs, including miR-210, induced by hypoxia in colon (HT29 and HCT116) and breast cancer (MCF7 and MDA-MB231) cell lines (Kulshreshtha et al., 2007). Later, miR-210 was recognized as the most prominently induced miRNA by hypoxia (Giannakakis et al., 2008; Camps et al., 2008). In the present study, we observed an elevation of circulating miR-210 in HHT patients with PAVMs. It is likely that PAVMs contribute to relative hypoxemic hypoxia, which stimulates cell production of miR-210 that is released into the circulation.

It has been recently reported that human umbilical vein endothelial cells produced miR-210, which was progressively increased after cell exposure to hypoxia (Fasanaro et al., 2008). Interestingly, miR-210 overexpression in endothelial cells stimulated the formation of capillary-like structures. As both endoglin and ALK1 are pro-angiogenic proteins, elevated focal expression of miR-210 in the lung under hypoxic condition, which stimulates angiogenesis, may be a compensatory mechanism for HHT gene haploinsufficiency.

Only a few studies designed to investigate possible biomarkers for HHT have been reported. Fernandez-L et al. identified several angiogenic genes (e.g. eNOS, angiopoietin-2) down-regulated in endothelial cells from HHT patients by gene expression microarrays. They further validated a decreased level of angiopoietin-2 in plasma samples from HHT patients (Fernandez-L et al., 2007). Ojeda-Fernandez et al. also observed a reduced plasma level of angiopoietin-2 as well as a decrease in soluble endoglin in HHT patients (Ojeda-Fernandez et al., 2010). Giordano and colleagues demonstrated that serum levels of VEGF in HHT children without AVMs were significantly lower than those with AVMs and normal controls (Giordano et al., 2009).

Our findings that circulating miR-210 levels were significantly elevated in HHT patients with PAVMs suggest that circulating miR-210 might provide a new biomarker for screening of HHT patients to identify those with PAVMs. If validated in a larger clinical series, the measurement of circulating miR-210 will provide a rapid, inexpensive, safe, and relatively non-invasive screening test for the diagnosis and follow-up of PAVMs in patients with HHT.

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Declaration of interest

The authors report no declaration of interest.

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