Circulating miRNAs from Dried Blood Spots are Associated with High Altitude Sickness

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Abstract

Circulating miRNAs isolated from dried blood spots (DBS) were found to be associated with high altitude sickness (HAS) patients in Tibet. HAS arises from two different diseases which are acute (AMS) and chronic (CMS) mountain sickness. Circulating miRNAs differences were found between AMS Han Chinese patients and normal Han controls and between CMS Tibetan Chinese patients and normal Tibetan controls. HAS arises from hypoxia which afflicts some high altitude inhabitants or visitors and not others. The difference results from each individual’s genetic makeup where hypoxia related genes have been shown to be a major contributor to these sicknesses. Several fold changes increases (up regulation) were found in the hypoxia associated miRNAs let-7f-5p, miR-9-5p, miR-19a-3p, miR-23a-3p, miR-98-5p, miR-125a-5p, miR-181b-5p, mir-202-3p, miR-372, miR-381-3p, miR-519d, miR-520d-3p, and miR-656 for both HAS groups compared to their controls. Other miRNAs (miR-19a-3p, 302c-3p and 875-3p) were found to be up regulated in one HAS group and down regulated in the other HAS group indicating the genetic differences between the two sickness groups.

Keywords: Circulating miRNAs; Dried blood spots; High altitude sickness

Introduction

MicroRNAs (miRNAs) are 18- to 25-nucleotides, non-coding RNA molecules that regulate the expression of many genes. Since their discovery, miRNAs have been found to regulate a variety of cellular processes including apoptosis, differentiation and cell proliferation [1]. Circulating miRNAs are found in all compartments of the blood, including plasma, platelets, erythrocytes and nucleated blood cells [2]. These circulating miRNAs are found to be remarkably stable in plasma even under harsh conditions as boiling, low or high pH, long-term storage at room temperature and in multiple freeze-thaw cycles [2,3]. Interestingly, circulating miRNAs are protected from endogenous RNAse activity [3] and evidence is now accumulating that this protection is achieved by packaging of plasma miRNAs into microparticles (e.g. exosomes, microvesicles or apoptotic bodies [4,5]) by binding to RNA-binding proteins (e.g. Argonaute 2 and nucleophosmin 1 [6,7]) or by linkage to high-density lipoprotein (HDL) [8].

Acute mountain sickness (AMS) is very common in lowlanders who ascend from sea level to altitudes greater than 2600 meters and is characterized by headache, lightheadedness, breathlessness, fatigue, insomnia, anorexia, and nausea [9,10]. Symptoms begin two to three hours after ascent. The condition is generally self-limiting; most symptoms disappear after two to three days, although insomnia may persist [11].

AMS must be treated as an emergency; the illness will resolve if no further altitude is gained however in some cases descent to a lower altitude may be necessary in order to reverse the condition. The precise pathogenesis of AMS is not well understood, but hypoxia which affects the regulation of angiogenesis [12] and erythropoietin [13] is likely to be a major factor [14-16].

Chronic mountain sickness (CMS) is characterized by polycythemia and severe hypoxemia, which is reversible upon descent from high altitudes [17,18]. Hematologic, neurologic, cardiac and respiratory symptoms are manifestations of the disease. The most common symptoms are bone and muscle pain, headaches, dizziness, dyspnea, insomnia, tinnitus, mental fatigue, and a loss of appetite. The severity of the condition increases with advancing age [19]. CMS is a syndrome resulting from the loss of human adaptation to high altitude and can occur in permanent residents residing in this environment [20,21]. The concept of collecting whole blood obtained from a finger prick and blotting it onto filter paper for use in screening of metabolic diseases has been available since 1963 [22]. This novel approach of blood collection has led to the population screening of newborns and other clinical testing [23,24]. The collection of whole blood samples on paper, known as dried blood spots (DBS), offers a simple sample collection method for storage or transfer of samples with reduced infection risk from various pathogens [25].

It has been demonstrated that miRNAs can be obtained from dried serum spots (DSS) [26]; however, it has not been shown that miRNAs can also be recovered from DBS. The preservation of miRNAs as DSS under various temperature and storage conditions did not adversely affect the analytical results of miR-16c [26] nor would we expect other miRNAs to be affected. The advantage of using a DBS over a DSS as a collection method is that DBS are obtained from a finger prick while...
DSS samples are collected after the bloods components are separated. The purpose of this preliminary study is two-fold, to determine if circulating miRNAs can be obtained from DBS and if so, to determine if the miRNAs can be used to detect genetic differences between sick and health individuals. In the second component of the study we looked for an association between circulating miRNAs and known hypoxia genes [27-29] by identifying fold changes differences in the circulating miRNAs between the HAS and healthy individuals.

Materials and Methods

Study groups

The Chinese ethnic groups studied were the Han who are considered upward migrants from low altitudes, and the Tibetans who are high altitude natives. AMS was studied in association with the Han while CMS was studied in association with the Tibetans resulting in two different HAS groups compared with their respective ethnic controls. All HAS patients in this study had been hospitalized and diagnosed at the Lhasa People Hospital (Tibet, China at 3, 658 M above sea level) from 2002 to 2008. The CMS patients and Tibetan controls normally live at 3600-4400 M. AMS was diagnosed by using the current consensus of mountain sickness in Tibet (Diagnosis and Therapeutics for Mountain Sickness, Xizang Autonomous Region), which is in accord with the Lake Louise scoring system [30]. The Lake Louise consensus on the definition and quantification of altitude illness [30] was the Qinghai diagnostic criteria for measuring CMS. We sampled Han AMS patients from the hospital with symptoms of acute pulmonary edema as diagnosed by a cough accompanied with pink frothy sputum. Moist or bubbling rales in the lungs was suggestive of pulmonary oedema, showing a characteristic shadow on chest X-rays. In addition to the characteristic symptoms of severe acute mountain response, acute cerebral edema was diagnosed by ataxia, disturbance of consciousness or coma, abnormal plantar reflexes and papilledema. The AMS Han patients were new comers from the low land and acquired the illness within two days after arriving at the higher altitude of Tibet. We also sampled Tibetan CMS patients as diagnosed by erythropoiesis, pulmonary hypertension and/or high arterial blood pressure, right ventricular hypertrophy or right and left ventricular hypertrophy. Patients with other diseases having similar clinical manifestations were excluded. Healthy Tibetan and Han people from the Lhasa area were randomly selected to serve as control subjects. All patients and controls sampled in the study signed an informed consent approved by the Human Ethics Committee of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and the study was approved by the appropriate institutional review board.

Sampling

DBS were prepared from whole blood obtained by a finger prick and blotted onto Whatman 903 filter paper. The DBS consisting of 50-100 μl of peripheral blood per spot were collected at the Lhasa People hospital during the Tibetan high altitude climbing period between early November, 2007 and late April, 2008. The DBS have been stored at the University of Washington in a -80°C freezer since the collection period. DBS for the controls were also collected during this period from unaffected Han and Tibetan Chinese that were determined to be in good health upon physical examination by doctors at the Hospital. Two circular DBS of 1.5 cm in diameter were used in the miRNA study per individual. As test subjects we choose DBS from three AMS and CMS patients and from three Han and Tibetan control individuals.

Physiological measurements

Heart rate (Hr) was measured by an electrocardiograph. Blood pressure (BPs/d) was measured by an automatic sphygmomanometer (China Jiangsu Diving Medical Equipment Co., LTD). The arterial oxygen saturation (SaO2) level measured on all participants of the study was conducted with a pulse oximeter device (Nonin Medical, Inc. Plymouth, MN USA, and Model 9500). All measurements were made at the Lhasa People Hospital, Tibet (3, 658 M altitude).

miRNA methods

DBS were cut into small pieces and placed in 1.5 ml of QIAzol (QIAGEN, Cat #79306). The samples were bead homogenized using the TissueLyser (QIAGEN, Cat # 85300). Excess pulp was removed from the homogenate and the lysate was transferred to a new 2ml Eppendorf tube. Three hundred microliters of chloroform was added to the lysate. Total RNA, including small RNAs, was then purified using miRNEasy mini kit (QIAGEN, Cat # 217004) according to the manufacturer's instructions. Final elution was done using minElute spin columns from the (QIAGEN, Cat # 1026497) 14 μl of nucleic-free water. Fifty nanograms of purified total RNA was reverse transcribed using the miScript II RT Kit (using miScript HiSpec Buffer provided in the kit, QIAGEN Cat # 218160) according to the manufacturer's instructions. Five μl of diluted cDNA for each sample was pre-amplified using miScript PreAMP PCR Kit (QIAGEN, Cat # 331451) in combination with miScript PreAmp inflation Primer mix (QIAGEN, Cat # MBHS105Z) according to the manufacturer's instructions. The pre-amplified cDNA was used for real-time PCR analysis using human inflammation miRNA PCR Array (QIAGEN, Cat #MHIS105ZD) and the miScript SYBR Green Kit (QIAGEN, Cat # 218073). Each inflammation miRNA PCR Array contain two miRNA isolation controls, six housekeeping snRNAs miScript PCR controls, and two miRNA reverse transcription controls and two positive PCR controls. The BioRad CFX96 real-time PCR instrument with a 96 well block was used for amplification. CFX manager software was used to generate the raw data (Ct values). The baseline was set from cycle 3 to 15 and threshold was set to 0.2. A table of Ct values was exported and uploaded onto miScript miRNA PCR Array Web-based software (http://pcrdatalanalysis.sabiosciences.com/mira/arrayanalysis.php). The cutoff gene expression values for inclusion in these analyses consisted of a fold change value of 2.0 or greater.

miRNA target genes

The miRNAs were associated with potential target genes using the miRNA-DISTILLER program which is an application to compile miRNA data from public databases [31]. The program uses the TargetScan 6.2 [32,33] microcosm [34,35] and miRDB [36] miRNA databases for calculating the respective miRNA intersections in obtaining the highest likely score for given target genes. From the DISTILLER program a list of miRNAs is generated along with their percent occurrence associated with potential hypoxia target genes (Supplement 1). A list of potential miRNA hypoxia target genes and their biological function are also provided (Supplement 2).

Results

The gender, age, medical condition and physiological parameters for the HAS patients and their normal controls are recorded in table 1. The physiological parameters arterial oxygen saturation levels (SaO2), blood pressure (BPs/d) and heart rate (Hr) were taken for each individual. In this preliminary study, we selected individuals of each sex, various ages and medical conditions that are representative of the two forms of HAS.
The outcome of the study indicates that circulating miRNAs can be obtained from DBS and that some miRNAs provide several fold change differences between the HAS and healthy individuals (Tables 2 and 3). The study revealed 24 up-regulated and 4 down-miRNAs in the AMS patients compared with the healthy Han controls while 48 were up-regulated in the CMS patients compared with the healthy Tibetan controls and 7 were down-regulated. miRNAs that are up and down regulated for the two HAS Chinese groups are provided in the tables. From the list, the miRNAs that are associated with hypoxia are emphasized in bold lettering while the miRNA associated with angiogenesis is emphasized in italic lettering. Several fold changes increases (up regulation) were found in the associated miRNAs let-7f-5p, miR-9-5p, miR-19a-3p, miR-23a-3p, miR-98-5p, miR-125a-5p, miR-181b-5p, miR-202-3p, miR-372, miR-381-3p, miR-519d, miR-520d-3p, and miR-656 for both HAS groups compared to their controls. Other miRNAs (miR-19a-3p, miR-302c-3p and miR-875-3p) were found to be

**Table 1:** Physiological parameters of the patients used in the miRNA study. The two ethnic groups studied are the Han and Tibetan Chinese. AMS is acute mountain sickness and CMS is chronic mountain sickness. Where health conditions are acute cerebral edema (ACE), acute pulmonary edema (APE), chronic high altitude polyglobulism (CR), chronic high altitude heart disease (CH) and CRH is CR + CH.

| Individuals sampled | Chinese Ethnic Group | Age (Years) | Sex | Health Condition | O₂ (mmHg) | BPd (mmHg) | Hr (bpm) |
|---------------------|---------------------|-------------|-----|------------------|-----------|------------|----------|
| AMS1                | Han                 | 30          | Male| APE              | 78        | 110        | 80       |
| AMS2                | Han                 | 42          | Male| APE,ACE          | 76        | 130        | 80       |
| AMS3                | Han                 | 27          | Male| APE              | 66        | 80         | 50       |
| Normal Han 1        | Han                 | 35          | Female| Normal         | 89        | 100        | 70       |
| Normal Han 2        | Han                 | 26          | Male| Normal           | 91        | 120        | 80       |
| Normal Han 3        | Han                 | 23          | Female| Normal         | 91        | 100        | 60       |
| CMS1                | Tibetan             | 46          | Female| CR             | 85        | 150        | 120      |
| CMS2                | Tibetan             | 50          | Male| CR              | 85        | 150        | 110      |
| CMS3                | Tibetan             | 60          | Female| CRH            | 78        | 160        | 120      |
| Normal Tibetan 1    | Tibetan             | 25          | Female| Normal         | 93        | 110        | 70       |
| Normal Tibetan 2    | Tibetan             | 42          | Female| Normal         | 94        | 90         | 60       |
| Normal Tibetan 3    | Tibetan             | 35          | Male| Normal          | 94        | 110        | 80       |

**Table 2:** miRNA regulatory change in the AMS patient group compared to the Han Chinese control. miRNA cluster or family, function, identification (ID) and fold change. Bold lettering indicates the miRNAs found in both HAS groups.

| miRNA Group Cluster or Family | miRNA Function Regulator of Biological Process | Mature MiRNA ID | Fold Change Regulation Up |
|------------------------------|-----------------------------------------------|-----------------|---------------------------|
| miR-17-92                    | angiogenesis, angiogenesis                     | hsa-miR-19a-3p  | 6.1652                    |
| miR-29 family                | Replicative senescence                         | hsa-miR-29c-3p  | 4.6325                    |
| miR-374 family               | hypoxia, oxidative stress                      | hsa-miR-374a-5p | 4.5004                    |
| miR-181 family               | hypoxia, oxidative stress                      | hsa-miR-181b-5p | 4.4315                    |
| miR-15b.16a.20               | angiogenesis, angiogenesis, hypoxia            | hsa-miR-19a-5p  | 4.3794*                   |
| miR-23 family, miR-519 family| hypoxia, suppresses growth, unknown            | hsa-miR-23a-3p  | 3.6372*                   |
| miR-30 family                | hypoxia, targets NF-κB and TGF-β signaling     | hsa-miR-30b-5p  | 3.0973                    |
| miR-520/373 family           | hypoxia, protein kinase activity               | hsa-miR-30e-5p  | 2.6995                    |
| Let-7 family                 | cell survival and proliferation                | hsa-let-7i-5p   | 2.4962*                   |
| miR-371-373                  | Wnt/β-catenin-signaling, hematopoiesis          | hsa-miR-372     | 2.4536                    |
| miR-125 family               | induces apoptosis, angiogenesis, cell proliferation, inflammatory response enhances TGFβ signaling | hsa-miR-125a-5p  | 2.3063                    |
| miR-181 family               | hypoxia, oxidative stress                      | hsa-miR-181a-5p | 2.0649                    |
| miR-125 family               | hypoxia                                        | hsa-miR-125b-5p | 2.0366*                   |
| miR-302-367                  | iPSC reprogramming                             | hsa-miR-302c-3p | -2.4249                   |
| miR-7 family                 | cell survival and proliferation                | hsa-let-7i-5p   | -4.415                    |
| miR-519 family               | hypoxia                                        | hsa-miR-524-5p  | -7.0955                   |
| miR-195-5p                   | hypoxia                                        | hsa-miR-195-5p  | -41.9591                  |

*p<0.05 significance of fold change
### miRNA Regulatory Change of the CMS Patient Group Compared to the Tibetan Chinese Control

| miRNA Group Cluster or Family | miRNA Function Regulator of Biological Process | Mature miRNA ID | Fold Change Regulation |
|------------------------------|-----------------------------------------------|-----------------|------------------------|
| miR-302-367                  | stemness                                       | hsa-miR-302b-3p | 13.3023                |
| miR-449 family               | NFκB/IκBα expression                           | hsa-miR-449a    | 12.9344                |
| miR-513 family               | TNF-α expression                               | hsa-miR-454-3p  | 11.3248                |
| miR-371-373                  | B7-H1 expression                               | hsa-miR-513b    | 10.0906                |
| Let-7 family                 | cell survival and proliferation                | hsa-miR-211-5p  | 9.9034                 |
| miR-520/373 family           | NF-κB-inducing kinase (NIK).                   | hsa-miR-520e    | 9.4078                 |
| miR-130 family               | B7-H1 expression                               | hsa-miR-524-5p  | 8.8707                 |
| miR-519 family               | senescence                                     | hsa-miR-519c-3p | 8.1714                 |
| Let-7 family                 | cell survival and proliferation                | hsa-miR-71-5p   | 8.8678                 |
| miR-181 family               | oxidative stress                               | hsa-miR-9-5p    | 8.0745                 |
| Let-7 family                 | cell survival and proliferation                | hsa-miR-98-5p   | 5.8264                 |
| miR-302-376                  | ROS-sensitive                                  | hsa-miR-302a-3p | 6.7353                 |
| miR-371-373                  | Wnt/b-catenin-signaling                        | hsa-miR-373-3p  | 6.0724                 |
| miR-130 family               | angiogenesis                                   | hsa-miR-130b-3p | 6.2714                 |
| Let-7 family                 | cell survival and proliferation                | hsa-miR-655     | 6.0129                 |
| miR-181 family               | unknown                                        | hsa-miR-656     | 6.1636                 |
| Let-7 family                 | hypoxia                                        | hsa-miR-181d    | 5.5382                 |
| miR-23 family                | angiogenesis                                   | hsa-miR-23b-3p  | 5.3805                 |
| miR-519 family               | growth suppression                             | hsa-miR-656     | 4.9725                 |
| miR-155,-16,-20              | anti-angiogenesis                              | hsa-miR-15b-5p  | 4.6767                 |
| miR-23 family                | angiogenesis                                   | hsa-miR-23a-3p  | 4.6418                 |
| miR-29 family                | angiogenesis                                   | hsa-miR-29b-3p  | 4.4824                 |
| miR-250/373 family           | NF-κB and TGF-β signaling pathways             | hsa-miR-520d-3p | 4.1134                 |
| miR-548 family               | signaling pathways                             | hsa-miR-548c-3p | 4.0322                 |
| miR-519 family               | growth suppression                             | hsa-miR-519d    | 3.9649                 |
| miR-302-367                  | IPSC reprogramming                             | hsa-miR-302c-3p | 3.9104                 |
| Let-7 family                 | hypoxia                                        | hsa-miR-875-3p  | 3.8722                 |
| miR-548 family               | signaling pathways                             | hsa-miR-548e    | 3.2276                 |
| miR-130 family               | angiogenesis                                   | hsa-miR-497-5p  | 3.0667                 |
| miR-449 family               | angiogenesis                                   | hsa-miR-449b-5p | 2.8544                 |
| miR-381-3p                   | adipogenesis and cell proliferation            | hsa-miR-381-3p  | 2.8158                 |
| miR-125 family               | apoptosis                                       | hsa-miR-186-5p  | 2.7067                 |
| miR-301 family               | cell proliferation                             | hsa-miR-301a-3p | 2.6357                 |
| miR-301 family               | angiogenesis                                   | hsa-miR-101-3p  | 2.242                  |
| miR-17-92                    | angiogenesis                                   | hsa-miR-301b    | 2.1414                 |
| miR-17-92                    | angiogenesis                                   | hsa-miR-19a-3p  | 2.5077                 |
| miR-17-92                    | cell proliferation                             | hsa-miR-19b-3p  | 2.5303                 |
| miR-17-92                    | adipogenesis and cell proliferation            | hsa-miR-145-5p  | 3.5363                 |
| miR-545-3p                   | unknown                                        | hsa-miR-545-3p  | 4.1717                 |

*p<0.05 significance of fold change.

*Bold lettering indicates the miRNAs are found in both HAS groups.*
up regulated in one HAS group and down regulated in the other HAS group indicating the genetic differences between the two types of illness. There were more miRNAs up and down regulated for CMS study group than there were for AMS group of individuals. This may be due to the nature of the sickness. AMS occurs in climbers within a few days of arrival at high altitudes and therefore may involve fewer affected genes then found in patients with CMS, which is an illness that manifests its self with age [37,38] and thereby reflects a broader array of genes. Eight of the first nine miRNAs in the AMS patients up-regulate either angiogenesis or hypoxia genes while the most down regulated miRNA (miR-195-5p) also regulates hypoxia genes (Table 2, Supplements 1 and 2) which possibility counter balances the up-regulation surge in hypoxia genes. The most up-regulated miRNAs in CMS patients involve signaling pathways (e.g., NFXb, TNFα, B7-H1, Wnt/β-catenin) followed by miRNAs that up-regulate hypoxia and angiogenesis while the most down-regulated miRNAs involve genes that regulate angiogenesis and cell proliferation (Table 3, Supplements 1 and 2). Only four (let-7f-5p, miR15a-5p, -23a-3p and -125b-5p) of the 24 up-regulated miRNAs were found to be statistically significant (p<0.05) for the AMS group of individuals. Of these miR15a-5p is an angiogenesis regulator; let-7f-5p regulates cell survival and proliferation while miR23a-3p and miR125b-5p regulate hypoxia genes (Table 2, Supplements 1 and 2). The only miRNA that was found to be significant among the CMS group of individuals was miR-186-5p which regulates apoptosis genes (Table 3, Supplements 1 and 2). We believe the lack of statistical significance in this preliminary miRNA study of HAS patients is because of the diversity of sample types selected for the study (e.g., gender, age and medical illness, Table 1). In future studies a more channeled focus should be taken (e.g., one gender, same age and same medical illness) for each HAS investigation [39]. Other miRNAs in tables 2 and 3 not apparently associated with HAS appear to be involved with aspects of gene regulation such as cell survival and proliferation, signaling or unknown function (Tables 2 and 3). The most up regulated miRNA (miR-302b-3p) for the CMS group is involved in generating new stem cells or cell proliferation (Table 3). The miRNAs miR-16-3p, miR-1-44-3p, miR-188-5p and miR-362 have previously been associated with erythroid differentiation [40,41] while the let-7 family, miR-16, miR-92b-5p, miR-130a, miR-296-3p and miR-378a have been shown to be associated with angiogenesis. A list of the miRNA potential hypoxia target genes were obtained from TargetScan Human 6.2 [32] and the miRNA-DISTALLER program [31] and is found in supplement 1, while the target gene function can be found in supplement 2. Also listed with the target genes in supplement 1 is the percent occurrence of each miRNA associated with the target gene. As can be seen from this supplement, miRNAs regulate multiple target genes and any target gene can be regulated by multiple miRNAs. Since in this study we are only focusing on hypoxia related miRNAs and target genes, the target genes in the supplement are by no means complete for each miRNA.

Discussion

The two-fold purpose of this preliminary investigation revealed that circulating miRNAs can be obtained from DBS after many years of storage at -80°C and that the miRNAs can be used as biomarkers in discriminating between normal and sick patients. In the only other study like this, it was reported that miRNAs from DSS remained unchanged after five months when stored at -80°C [26]. The integrity of miRNAs obtained from DBS that have been stored for many years needs further investigation with regard to quality control issues especially the stability of miRNAs in DBS over time. Since this is only a preliminary study, such issues will have to be addressed in a more in depth DBS study of miRNAs which is beyond the scope of the present study. In this report, we find that miRNAs can be obtained from DBS and that the miRNA data when compared between a normal (control) group and a HAS patient group revealed changes in miRNAs that regulate hypoxia genes associated with HAS.

Hypoxia leads to an increase in the hypoxia-inducible factor (HIF) activity that induces the expression of genes which mediates the adaptive responses through glycolytic enzymes, hemoxxygenase, vascular endothelial growth factor (VEGF) and erythropoietin (EPO) [28, 42]. From the table we find that HIF-1α is regulated by miR-93-5p, miR-519c-3p and miR-519d, miR-548e and miR-656 and its inhibitor HIF-1αN is regulated by the let-7 family and many others. The egl nine homolog 1 (EGLN1) gene is a key oxygen sensor that negatively regulates the activity of HIF-1α and itself is regulated by miR-15a-5p, miR-130a-3p, miR-21-5p, miR-93-5p, miR-372, miR-513b, miR-510d, miR-520d-3p, miR-520e, miR-524-5p, miR-590-5p and miR-607. The other EGLN genes (2 and 3) which provide the same function in different tissues were also found to be regulated by miRNAs in the HAS patients (Tables 1 and 2). The endothelial Per-Arnt-Sim (PAS) gene (EPAS1) which encodes hypoxia-inducible-factor-2 alpha (HIF2A) is a transcription factor which is also involved in the response to hypoxia. EPAS1 is regulated by miR-23a, b-3p, miR-340-5p, miR-548c-3p, miR-548e and miR-875-3p. The HIF-3α gene is regulated by miR-29b and c-3p, miR-98-5p, miR-300, miR-381-3p, miR-519c-3p, miR-519d and miR548c-3p.

VEGF is a potent permeability factor subject to hypoxic regulation and has been shown to be an important component of the pathogenesis of high altitude adaptation and sickness [43-45]. The VEGF signaling pathway is a family of key regulators in critical physiological and pathological angiogenesis [46] including tissue growth, wound healing, rheumatoid arthritis, proliferative retinopathies, cardiovascular disease and cancer [47]. Among all family members, VEGFA is the most potent and best known angiogenic protein and exerts its biologic effect through interaction with cell-surface receptors which triggers a cascade of downstream dimerizations and phosphorylations [48]. It can be seen from supplement 1 that the VEGFA gene is regulated by miR-16-2, miR-29b, c-3p, miR-186-5p, miR-195-3p, miR-300, miR-374a-5p, miR-381-3p, miR-519d, miR-549c-3p and miR-549e while the VEGFB gene is regulated by miR-125a-5p and VEGFC gene is regulated by miR-511, miR-513b, miR-875-3p and miR-1324.

EPO is a glycoprotein hormone that controls erythropoiesis, or red blood cell production. EPO has a range of actions including vasocostriction-dependent hypertension, stimulating angiogenesis, and inducing proliferation of smooth muscle fibers. Under hypoxic conditions, the kidney will produce and secrete erythropoietin to increase the production of red blood cells by targeting colony forming unit-E, pro-erythroblast and basophilic erythroblast subsets in the differentiation. We have previously reported that red blood cell count, hematocrit and hemoglobin concentration are significantly increased in our CMS patients compared to their Tibetan control group [27]. In this study we found EPO to be regulated by miR-125a, b-5p and the miRNAs are up regulated in both the AMS and CMS patients (Tables 1 and 2).

The phosphatase and tensin homolog (PTEN) which regulates the PI3K-AKT/PKB signaling pathway is itself regulated by miR-19a, b-3p, miR-130a, b-3p, miR-301a-3p, miR-454-3p, miR-511, miR-513b, miR-519c-3p, miR-519d, miR-548e and miR-875-3p in our HAS patients (Tables 1 and 2). PTEN antagonizes the PI3K-AKT/PKB signaling pathway and best know angiogenic protein and exerts its biologic effect through interaction with cell-surface receptors which triggers a cascade of downstream dimerizations and phosphorylations [48]. It can be seen from supplement 1 that the VEGFA gene is regulated by miR-16-2, miR-29b, c-3p, miR-186-5p, miR-195-3p, miR-300, miR-374a-5p, miR-381-3p, miR-519d, miR-549c-3p and miR-549e while the VEGFB gene is regulated by miR-125a-5p and VEGFC gene is regulated by miR-511, miR-513b, miR-875-3p and miR-1324.

The phosphatase and tensin homolog (PTEN) which regulates the PI3K-AKT/PKB signaling pathway is itself regulated by miR-19a, b-3p, miR-130a, b-3p, miR-301a-3p, miR-454-3p, miR-511, miR-513b, miR-519c-3p, miR-519d, miR-548e and miR-875-3p in our HAS patients (Tables 1 and 2). PTEN antagonizes the PI3K-AKT/PKB signaling pathway by dephosphorylating phosphoinositides
and thereby modulating cell cycle progression and cell survival. The phosphatidylinositol 3-kinase (PI3K)/AKT pathway plays a key role in numerous cellular functions including proliferation, adhesion, migration, metastasis, and survival [49] as well as angiogenesis. The PI3K/AKT pathway modulates the expression of angiogenic factors such as nitric oxide and angiopoietins [50]. AKT3-1 are three isoforms of the AKTs which are major downstream targets of growth factor receptor tyrosine kinases that signal through PI3K [51]. All three isoforms are regulated by miRNAs in both forms of HAS (Tables 2 and 3). AKT1 is regulated by miR-449a, miR-520d-3p and miR-656, while AKT2 is regulated by miR-29b, c-3p, miR-98-5p, miR-181d, miR-449a, miR-511 and miR-875-3p and AKT3 is regulated by miR-15a, b-5p, miR-125a-3p, miR-145-5p, miR-181a, b-5p, miR-181d, miR-195-5p, miR-424-5p, miR-524-5p, miR-548e and miR-607 and miR-655. Angiotensinogen (AGT) which is a potent regulator of blood pressure, body fluid and electrolyte homeostasis is regulated by miR-181d, miR-512b and miR-548c-3p in CMS patients (Table 2). The angiotensin II receptor, type 1 (AGTR1) is a potent vasopressor hormone that controls blood pressure and volume in the cardiovascular system is regulated by miR-181a, b-5p and miR-449a. The angiotensin II receptor, type 2 (AGTR2) is a receptor for angiotensin II and is an intergal membrane protein that is highly expressed in brain (adrenal medulla) and is regulated by several miRNAs including miR-93-5p, miR-302a, b and c-3p, miR-372, miR-373-3p, miR-519d, miR-520d-3p, miR-520e, miR-524-5p, miR-607, miR-655 and miR-875-3p.

The angiopoietin protein genes (ANGPT1 and 2) play an important role in vascular development and angiogenesis and have hypoxia-induced expression in endothelial cells. Both genes are regulated by multiple miRNAs in the two forms of HAS. The ANGPT1 gene is regulated by miR-181d, miR-211-5p, miR-374a-5p, miR-548e and miR-656, while the ANGPT2 gene is regulated by let-7c, miR-19a, b-3p, miR-125a-5p, miR-145-5p, miR-181d, miR-340-5p, miR-511, miR-607, miR-655 and miR-656. The ANGPT2 protein is a secreted glycoprotein with homology to the angiopoietins that may exert a function on endothelial cells through autocrine or paracrine action and its gene (ANGPT2L) is regulated by miR-875-3p.

Recently, it has been found that the epidermal growth factor receptor (EGFR) modulates miRNA maturation in response to hypoxia through the phosphorylation of argonuate 2 (AGO2) [52]. The argonuate family of proteins plays a role in RNA interference through the RNA-induced silencing complex (RISC). Here we find that miR-875-3p which is down regulated in AMS (Table 1) and up regulated in CMS (Table 2) is involved in the gene regulation of AGO2 (Supplements 1 and 2). We also find that miR-548c-3p which is up regulated in CMS (Table 2) is involved with the regulation of both AGO2 and EGFR (Supplements 1 and 2).

Other miRNAs and their target genes which are listed in tables 1 and 2 will not be discussed because that would be beyond the scope of this preliminary investigation. The purpose of this study was to demonstrate that circulating miRNAs can be isolated from DBS and that the miRNA data has meaningful value. The interpretation of the other miRNAs and their target genes will have to wait for thorough investigation of HAS using a more balanced and focused approach. Based on this preliminary report, there is a need for an in depth investigation of miRNA integrity associated with DBS and a more comprehensive study of miRNAs and HAS.

Conclusion

We show in this preliminary investigation that circulating miRNAs can be obtained from DBS after several years when stored at -80°C and that they may be used to differential between patients with HAS and healthy individuals. The study indicates that circulating miRNAs regulate many hypoxia related genes associated with HAS. Since it has never been reported that circulating miRNAs can be obtained from DBS, the methods provided in this study should be of value in clinical medicine and miRNA research.

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Appendices

Supplemental material is available for this article.

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