Symbiosis

www.symbiosisonlinepublishing.com
ISSN Online: 2377-4274

Research Article | International Journal of Genetic Science | Open Access

An In-Silico Argument for MicroRNAs Showing Pivotal Role in Susceptibility towards High Altitude Induced Venous Thrombo-Embolism (HA-VTE)

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Received: April 04, 2019; Accepted: May 08, 2019; Published: May 14, 2019

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Abstract

Background: Venous Thrombo-Embolism (VTE) may occur spontaneously upon climbing or prolonged stay at High Altitude (HA). VTE has complex multi factorial aetiology including acquired, inherited and environmental risk factors. Various studies show that environmental conditions prevailing at HA such as hypoxia, dehydration, hem concentration, use of constrictive clothing and enforced stasis because of severe weather may result formation of blood clots thus leading to thrombotic disorders. Early diagnosis of VTE is often difficult which delays treatment resulting in sudden deaths. Several recent reports have illustrated the role of differentially expressed micro RNAs (miRNAs) and their potential value in DVT susceptibility and diagnosis. Present in-silica analysis, aims to identify miRNAs playing critical role in VTE susceptibility and pathogenesis under hypoxic environment at HA.

Methods: Online databases were searched for recent reports that link different miRNAs with VTE and other coagulation disorders. Relevant references were handpicked and list of potential miRNAs was prepared. Target gene list for each miRNA was subjected to pathway analysis using online bio-informatics tools. We thus identified most potential miRNAs which target genes of critical VTE-linked and hypoxia-linked pathways. These selected potential miRNAs were further subjected to network analysis and gene ontology.

Results: We prepared list of 25 miRNAs reportedly linked to VTE. Pathway analysis of target gene list revealed 6 most potential miRNAs linked to VTE. On the basis of network analysis and gene ontology of miRNA target genes, we shortlisted 4 most promising miRNAs, hsa-miR-19b, hsa-miR-20a, hsa-miR-24-3p, hsa-miR-106b that could be involved in VTE susceptibility under hypoxic conditions.

Conclusion: This study provides insights into the causes of higher incidences of VTE occurrence at HA. It provides groundwork for future studies concerning VTE susceptibility biomarkers and treatment. Further in-depth exploration in this area is required to establish diagnostic and therapeutic values of these miRNA for VTE.

Keywords: Venous thrombo-embolism; Deep vein thrombosis; Pulmonary embolism; High Altitude; miRNA; Coagulation;

Introduction

Thrombo-Embolic Disorders (TED) comprising of both venous and arterial thrombosis together results in significant morbidity and mortality every year worldwide. It is the third leading cause of cardiovascular deaths, surpassed only by myocardial infarction and stroke (Heit et al. 2008) Venous Thrombo-Embolism (VTE) alone causes large number of deaths annually and the toll reaches to approximately 3lac deaths annually in US alone (Heit et al. 2008). Regardless of its initial cause, major driving factor for VTE is blood flow restriction or stasis (Bovill et al. 2011). Frequency of occurrence of Deep Vein Thrombosis (DVT) and Pulmonary Embolism (PE) is reportedly higher in sojourns travelling to High Altitude (HA) and soldiers posted at HA without any other co-existent risk factor (Kumar 2006; Anand et al. 2001; Khalil et al. 2010;Rathi et al. 2012). HA hypoxia is a path physiological condition wherein lesser number of oxygen molecules are available for breathing due to lowered atmospheric pressure and thus lowered concentration of oxygen is supplied to body tissues (Koh and Powis 2012). Hypobaric hypoxic environment prevailing at HA leads to increase in the viscosity of blood due to increase in pro-coagulatory factors (Pichler-Hefti et al. 2010), platelet aggregation, rise in fibrinogen levels and polycythemia, which further leads to thrombotic state (Vij, 2009). This implies that exposure to HA environment further results in hyper coagulable state. Many case reports of HA mountaineers suffering from thrombosis and eventually death have been documented (Bartsch, 2006).A 30 times higher risk of spontaneous VTE has been reported in young male soldiers with mean stay of over 10 months at altitudes from 3000 m to 6500 m (extreme HA) (Anand et al. 2001). However not much experimental evidence is available to prove thrombogenicity of HA and real time experiments to prove the same are a difficult task.

The only established clinical biomarker for DVT today is D-dimer test, however it has low specificity (Pulivarthi et al. 2014). D-dimer is a fibrin degradation product and its estimation

is the most widely used method for VTE diagnosis. Although, D-dimer assay is highly sensitive, it gives false positive results many times. Other imaging modalities available for PE diagnosis are expensive, require expertise and are not widely available (Khalil et al. 2010), especially at remote areas of HA.VTE therapy mainly includes anticoagulant treatment. Unfractionated Heparin (UFH) or Low Molecular Weight Heparin (LMWH) and vitamin K antagonists are mostly used for the treatment of VTE, with the aim to control thrombus formation or embolization and prevent recurrent VTE. However this conventional anticoagulation treatment has several disadvantages as it requires routine monitoring, frequent dose adjustments and has several unpredictable responses including excessive bleeding. Over the last years, new oral anticoagulant agents have been developed such as direct thrombin inhibitors, like dabigatran etexilate and direct factor Xa inhibitors like rivaroxaban, apixaban or edoxaban. Recently international clinical trials have been conducted for the use of Direct Oral Anticoagulants (DOACs) for treating VTE and preventing its recurrences, and the results have been very promising (Buller et al. 2013, Bauersachs et al. 2010, Agnelli et al. 2013). These new drugs are closely approaching markets as they are administered at fixed daily doses and do not require regular monitoring. However, efficacy of DOACs is yet to be demonstrated in some specific populations. A large number of research groups worldwide are working towards development of new safer and effective VTE treatment strategies which are convenient in administration and have predictable pharmacokinetics, pharmacodynamics that would permit a fixed dosing without requirement of regular monitoring.

Circulating endogenous micro RNAs (miRNA), a class of non-coding small RNAs that suppress their target genes at post transcriptional level (Bartel et al. 2004), could fill this lacunae as they have been proved to have potential diagnostic and therapeutic value in variety of complex cardiovascular diseases (Alessandra et al. 2010; Corsten et al 2010; Meyer et al. 2013). MiRNAs serve as key post-transcriptional regulators of gene expression and have been shown to play critical roles in key cellular processes such as stem cell differentiation, signalling, cell cycle control and apoptosis (Noman et al. 2016). Many recent studies have demonstrated their critical role in diseases like cancer and auto-immune disorders (Kulshrestha et al. 2007, Brown 2007, Greco et al. 2016). To assess potential of miRNAs to be used as biomarkers in VTE susceptibility and pathogenesis or as potential therapeutic agents for its treatment, we conducted an *in silico* study using publicly available web based bioinformatics tools. Candidate miRNAs demonstrated in various studies for their links with DVT and other vascular disorders were selected. We then applied various bio-informatics tools to narrow down to four miRNAs as most promising candidates for determining VTE susceptibility at HA and as potential targets that could be modulated for VTE treatment under hypoxic environment. Also, we have enlisted their commonly targeted genes that could mediate vascular functions at HA.

Methodology

This study is based on literature review of published data and its further in-silico analysis in order to gain insights into

Table 1: List of various potential miRNAs involved in venous thrombosis and their related factors: Selected from relevant published research articles

S. No.	miRNA	References
1	hsa-miR-126-3p/5p	Wang et al. 2012, Zametaki et al. 2010, de boer et al. 2013
2	hsa-miR-197	Willeit et al. 2013, Zampetaki et al. 2010, Karolina et al. 2012
3	hsa-miR-21-5p	Zampetaki et al. 2012, Cheng et al. 2010, Wang et al. 2014
4	hsa-miR-150	Carino et al. 2016
5	hsa-miR-24-3p	Lu-jia-Chen 2017
6	hsa-miR-145	Sahu et al. 2017
7	hsa-miR-29	Hatziapostolou et al. 2011, Fish et al. 2012, Van Roaij 2008
8	hsa-miR-409-3p	Fort et al. 2010
9	hsa-miR-18a	Teruel et al. 2011
10	hsa-miR-19b	Teruel et al. 2011, Zhang et al. 2011,Yu et al. 2013
11	hsa-miR-20a, hsa-miR-106b	Teruel et al. 2011
13	hsa-miR-421	Marchand et al. 2012
14	hsa-miR-30, hsa-miR-301	Patel et al. 2011
15	hsa-miR-335	Bao et al. 2017
16	hsa-miR-10b-5p,hsa- miR-320a/b, hsa-miR-424-5p, hsa-miR-423-5p, hsa-miR-103a-3p,hsa-miR-191-5p, hsa-miR-301a-3p, hsa-miR-199b-3p	Starikova et al. 2015
18	hsa-miR-136	Wang et al. 2016
19	hsa-miR-26a	Lizi et al. 2017

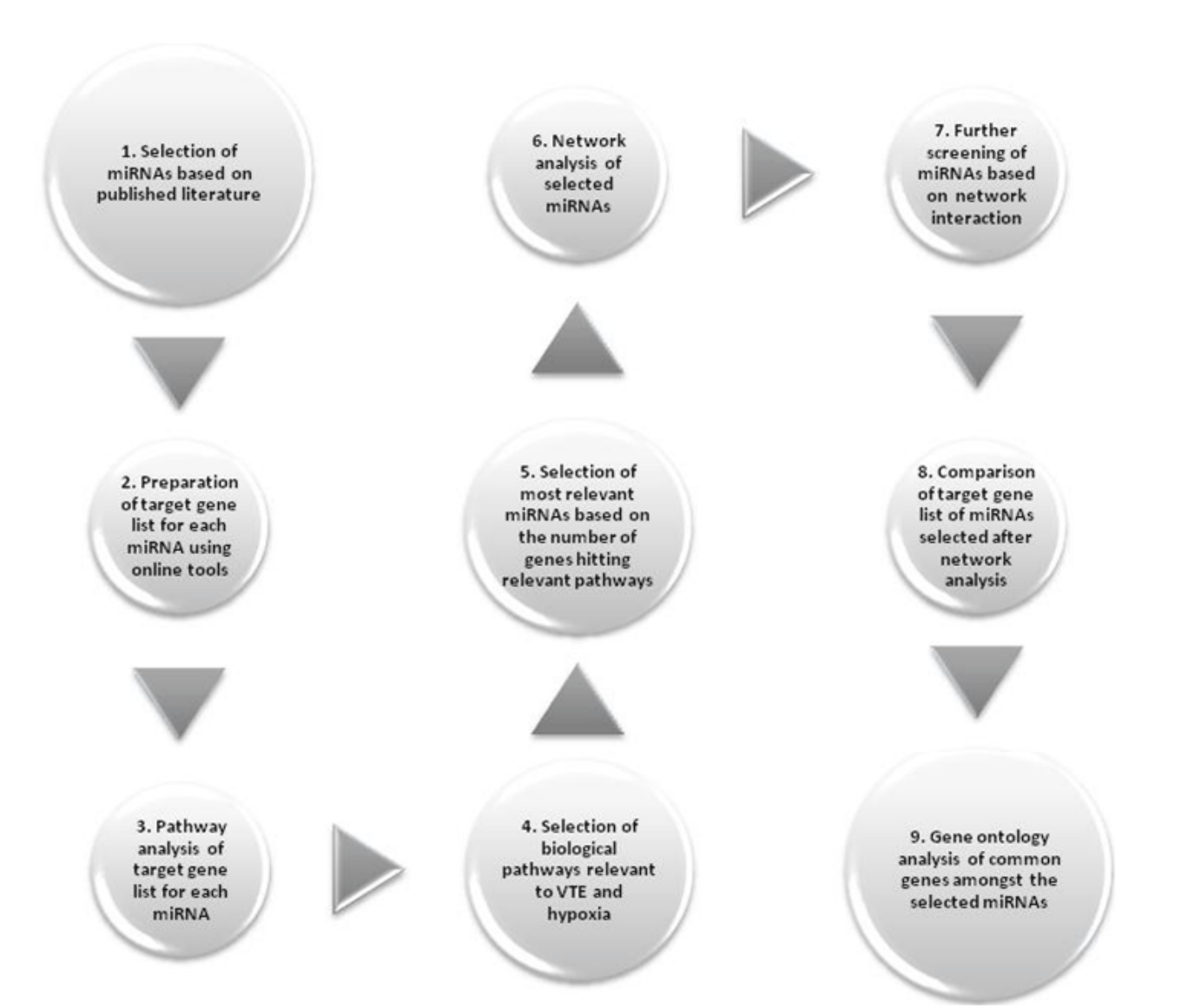


Figure 1: Summary of work-flow.

increased rates of occurrence of VTE at HA. No new patient data is presented in this study and hence ethical approval was not necessary. A summary of steps involved in the present in-silico study are given in figure 1.

Literature search for miRNAs associated with VTE and related complications

Data sources and retrieval

Exhaustive web researching was conducted for recent research articles published in databases Pub Med, Embase, Web of Science etc.. Articles linking various miRNAs directly with venous thrombosis and those linking miRNAs with its other associated risk factors such as angiogenesis, platelet production, factor X inhibition, fibrinogen and tissue factors production, SERPINE1 inhibition and PAI-1 induction (as detailed in table 1) were selected. Based on all the relevant studies that we could collect after web researching, a curated list of miRNAs with obvious involvement in venous thrombosis aetiology was prepared table 1.

Target identification

Two different databases; miRDB (Wong et al. 2015) and miRDIP (Tokar et al. 2018) were used to identify target genes of selected miRNA. miRDB includes 2.1 million predicted gene targets regulated by over six thousand miRNAs, whereas miRDIP provides nearly 152 million human microRNA-target predictions, collected across 30 different resources. The selection of target genes were made based on their target prediction score. This included both computational predictions as well as experimentally validated genes. To further study mechanistic insights into the role of miRNAs, target gene list for selected

miRNAs were obtained from both the databases. Target gene list obtained from each database were compared and overlapping genes were removed. The resultant number of target genes for each miRNA is enlisted in table 2. We used this target gene lists for downstream analysis such as pathway analysis and gene ontology.

Pathway analysis

Target gene list obtained for each miRNA was subjected to pathway analysis using Reactome database (https://reactome.org/PathwayBrowser/) (Croft et al., 2014, Fabregat et al. 2018) and Panther pathways (http://pantherdb.org/tools/index.jsp) (Mi et al., 2010). Results were compiled for a total of eight selected pathways, four pathways most relevant to VTE occurrence (VTE-linked pathways); (i) blood coagulation, (ii) hemostasis, (iii) platelet activation, signalling and aggregation and (iv) endothelin signalling and four pathways relevant to hypoxic adaptation of an individual (Hypoxia linked pathways); (i) hypoxia response via Hif activation, (ii) angiogenesis, (iii) apoptosis and (iv) homeostasis. These pathways were chosen as they are directly affected during VTE path physiology and during HA hypoxic exposure, respectively.

Network analysis

Six miRNAs out of twenty five that were found to regulate maximum genes linked to VTE and hypoxia sensitive pathways were selected for further analysis. These top six miRNAs were subjected to network analysis using web tool miRNet (http://www.mirnet.ca.). This web tool generated high quality miRNA-target interaction networks using various databases. In order to control the size of the large network while keeping the useful information intact, various network filtration criteria were applied. These filtering tools were based on topological measures such as degree, betweens and shortest path. Shortest path filter applied to all nodes except miRNA nodes proved to be the most optimum in this case. After applying filters, miRNAs connected with maximum nodes were selected from the miRNet networks and were again subjected to study of target-gene overlap between multiple miRNAs using miR Target Link Human.

Comparison of target genes

Target gene list of selected miRNAs were compared using Venn diagram analysis. Venn diagram was generated using online tool; *http://bioinformatics.psb.ugent.be/webtools/Venn/*. It showed the number of genes common for all selected miRNAs and also number of genes commonly shared amongst rest three or two miRNAs.

Functional gene annotation

The target genes which were common to all the selected miRNAs were subjected to functional gene annotation using "GO term enrichment" with online tool PANTHER (Huaiyu et al. 2016). This helped us to identify processes that were significantly enriched for each set of genes.

Results

MicroRNAs selected for study

All recent publications available online were thoroughly searched and scrutinized for selection of most potential or relevant miRNAsfor the present study. Those miRNAs found linked to VTE susceptibility directly were chosen along with other miRNAs that have been shown to play a role in platelet activation, angiogenesis, regulation of endothelial functions, Tissue Factor (TF) expression, production and regulation of fibrinogen and matrix proteins like collagens, elastin, other coagulation factors and cardiovascular diseases. Thus, a total of twenty five miRNAs from twenty five different studies were finally selected for further in-silico analysis. Table 1 elaborates the list of selected miRNAs, their proven function and the concerning references.

Identification of target genes of significant miRNAs

A single miRNA can potentially regulate several hundred to several thousands of transcripts. Several bio-informatics portals are available to predict miRNA targets, however they are mostly based on sequence similarity and other algorithms such as seed match, conservation, free energy, and site accessibility (Peterson et al. 2014); all the targets are not experimentally validated. Thus, in order to make our study more robust, we used two different online databases to search for miRNA-mRNA interactions. These were miRDB and miRDIP. The target genes thus obtained were selected based on their target prediction score. After removing the duplicate and unknown genes from each list, the number of genes targeted by each selected miRNA was found to be highly variable table 2.

Table 2: List of selected miRNAs and the number of their target genes: Target genes list has been prepared based on two online available target prediction tools. Number of overlapping genes obtained from different databases has been subtracted from the final number.

S. no.	MicroRNA	Number of gene targets
1	hsa-miR-126-3p/5p	782
2	hsa-miR-197	3997
3	hsa-miR-21-5p	956
4	hsa-miR-150	1245
5	hsa-miR-24-3p	1529
6	hsa-miR-145-5p	1124
7	hsa-miR-29a	1508

8	hsa-miR-409-3p	887
9	hsa-miR-18a	1005
10	hsa-miR-19b	2022
11	hsa-miR-20a	5117
12	hsa-miR-106b	2163
13	hsa-miR-421	1215
14	hsa-miR-30c-5p	1836
15	hsa-miR-301a-3p	1729
16	hsa-miR-335-5p	1024
17	hsa-miR-10b-5p	575
18	hsa-miR-320a/b	1817
19	hsa-miR-424-5p	2599
20	hsa-miR-423-5p	816
21	hsa-miR-103a-3p	2070
22	hsa-miR-191-5p	976
23	hsa-mir-199b-3p	1807
24	hsa-miR-136	3027
25	hsa-miR-26a	1485

Pathway analysis

Different micro RNAs from our data set recognized several gene targets. The target list obtained for each miRNA was subjected to pathway enrichment analysis using PANTHER pathways and Reactome pathway database. Although pathway enrichment analysis enlisted large number of pathways regulated by target genes of each miRNA, we focused ourselves to the pathways governing cascade of events in venous thrombosis and hypoxia. Our study included four key pathways involved in venous thrombosis such as blood coagulation, hemostasis, platelet function and endothelia signalling. Similarly, four pathways indicative of hypoxic stress were included namely, hypoxia response via Hif activation, angiogenesis, apoptosis and homeostasis. The number of target genes hitting these pathways from each miRNA target gene list, was extracted and has been represented in table 3. Six out of twenty five miRNAs (shaded in dark grey); hsa-miR-197, hsa-miR-24, hsa-miR-19b, hsa-miR-20a, hsa-miR-106b and hsa-miR-136 were shown to mediate larger number of genes involved in both VTE linked pathways and hypoxia-linked pathways. This converged our focus to these six miRNAs instead of twenty five, for further analysis. Also, notably there were four miRNAs (shaded in light grey); hsa-miR-29a, hsa-miR-320a, hsa-miR-424 and hsa-miR-103a, which did not hit more than three genes of blood coagulation pathway, however were largely mediating genes of other VTE linked and hypoxia linked pathways. Two miRNAs; hsa-miR-30c and hsa-miR-199b were ranking high in mediating pathways linked to VTE but not of hypoxia and similarly, two miRNAs; hsa-miR-150 and hsa-miR-26a ranked high in genes mediating pathways linked to hypoxic response not that of VTE.

Network analysis

Our next target was to study the molecular interactions of top six selected miRNAs with their target genes. This was done using online application miRNet (Fan et al. 2018, Fan et al. 2016). All the six miRNAs were taken together which generated large and complex regulatory network as shown in 2(a). Detailed study of the network properties was done such as between's centrality for the network, which measured the number of shortest paths going through the node taking into consideration the global network structure. All miRNAs in the network showed varying degree and between's centrality amongst them. When shortest path filter was applied to all network nodes except miRNA nodes, four miRNAs; hsa-miR-20a-5p, hsa-miR-106-5p, hsa-miR-19b-3p and hsa-miR-24-3p; were found to be at the centre of the network, showing maximum connected nodes (figure 2(a) & 2(b)).

Another interaction network of these four selected miRNAs was generated using another web based application miR Target Link (as shown in figure 3), which generated the set of genes overlapping amongst these four miRNAs. The colour of the genes in the image thus generated indicates the amount of interactions they are involved in. Genes that are targeted by two micro RNAs are coloured blue; strong interactions are marked by green figure 3.

Comparison of target gene list of selected miRNAs

In order to identify the overlapping gene entities amongst the four shortlisted miRNAs in a comprehensive manner, we compared the entire target gene list of these four miRNAs and generated Venn diagram. It showed 135 genes as common targets for all the four miRNAs. 76 gene targets were common for three miRNAs except for hsa-miR-106b; 501 genes were also common

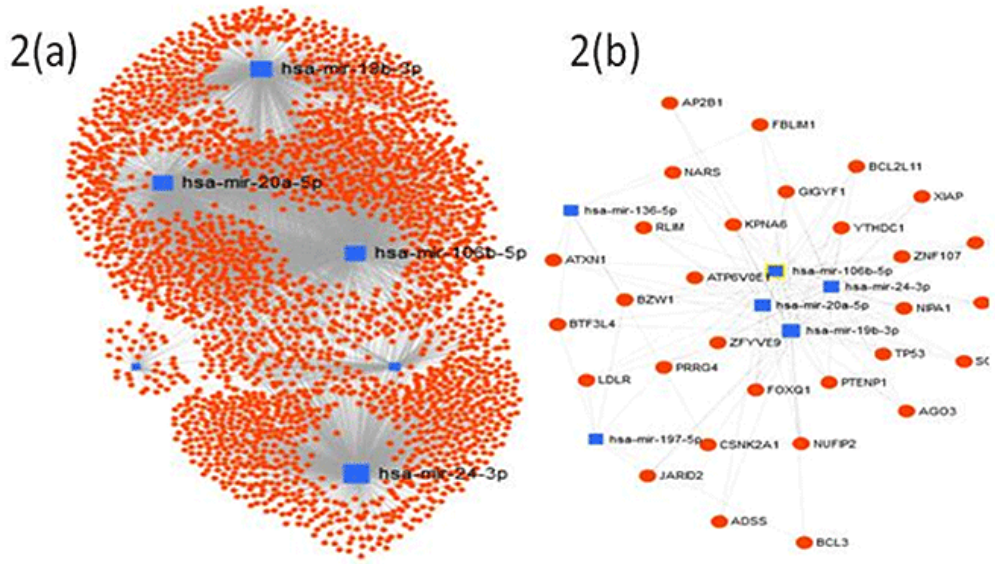


Figure 2: Network analysis **(a)** Gene regulatory network of six miRNAs selected after pathway analysis. 4 miRNAs; hsa-miR-19b, hsa-miR-20a, hsa-miR-106b and hsa-miR-24 showed highly dense connected nodes.
(b) Gene regulatory network after applying shortest path filter to all 6 miRNAs. 4 miRNAs, hsa-miR-19b, hsa-miR-20a, hsa-miR-106b and hsa-miR-24 were placed in the centre of the network showing highest connected nodes. Squares marked in blue in both the networks represent miRNAs whereas the orange dots represent the genes. Fine lines show the connections between the miRNAs and the genes.

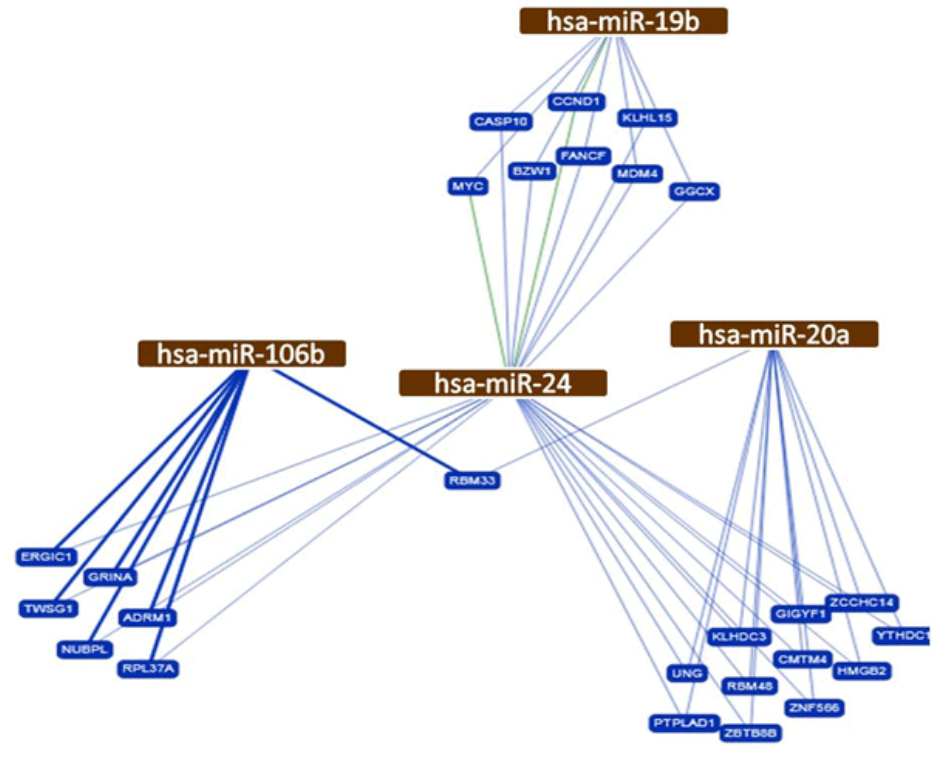
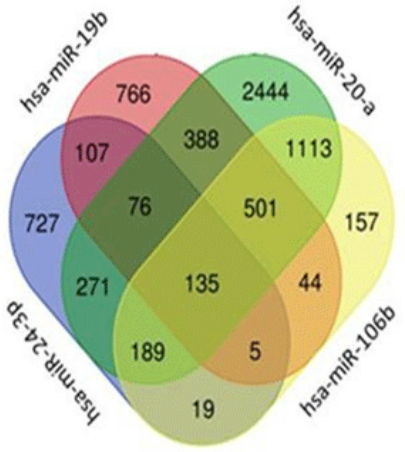


Figure 3: miRNA-mRNA Interaction network of top four selected miRNAs: Each miRNA (placed in brown boxes) common target genes (placed in blue boxes) and the connections amongst them are shown by blue lines. Genes that are targeted by two micro RNAs are coloured blue, strong interactions are marked by green.

to all except hsa-miR-24. Also, there were a large number of unique gene elements for each miRNA; 2444 for hsa-miR-20a; 727 for hsa-miR-24; 766 for hsa-miR-19b and 157 for hsa-miR-106b. The results have been summarized in figure 4.

Functional annotation (Gene ontology)

Gene Ontology (GO) function assigns Biological Processes (BP), Cellular Components (CC) and Molecular Function (MF). GO enrichment was done using online application of PANTHER classification system. This functional annotation of miRNA target genes categorized them into biological processes, molecular function and cellular component genes. Amongst top terms of biological processes included those belonging to biological regulation, response to stimulus, biological adhesion and signalling. Meanwhile cell component terms were enriched mainly in cell, organelle, membrane and protein-containing complex. Terms included in molecular function were primarily involved in binding, catalytic activity and transcription regulator activity figure 5.



Common elements (Total=135)

KBTBD8 GDA ATL2 VGLL3 FRS2 WDFY2 LIMD1 THRB KDM2A UBN2 FUT4 GMFB SH3PXD2A TANC2 PLEKHM3 ICA1L XIAP MKLN1 SPATS2L ITGA2 KIF1B RB1CC1 NTRK2 TACC1 KLHL15 MKL2 MCC MBNL3 RAP2A CHIC1 RASA1 TET1 NEK9 STX6 BNC2 AAK1 BTAF1 LPP WWC2 TGFB2 AP1G1 YOD1 DCBLD2 ACBD5 SCML2 NCEH1 AQP4 ENAH DYRK2 RNF2 PRR14L SATB2 PCDHA9 KLF12 EREG IPMK USP37 MTMR9 AGO1 BMPR2 S1PR1 ERBB3 NUP58 PLAG1 TP53INP1 PAK5 FRK HIF1A ANGEL2 CHD5 SETD7 PTP4A1 RLIM CASP2 SMAD5 CNOT6L KPNA3 ABI2 CNOT6 ASXL2 VTI1A ANTXR1 MARVELD3 PLEKHO2 ITGB8 FRMD4A KPNA4 PSD MED13 CSNK1G1 SGCD PURB ATXN1 BNIP3L DLC1 UBFD1 RNF8 RNF11 ETV1 GIGYF1 RGMB EXOC5 RAB8B PHLPP2 TGM2 SOCS6 CSF1 NPAT CASP8 NRP2 UHMK1 USP9X FURIN MYO5A ZNF217 HEG1 CLTC GAPVD1 AFF2 PPARA PTPRD BCL2L11 PCGF5 RAP1B NFIB RASSF2 GTDC1 SKIDA1 SRGAP3 RIMS2 CYB561D1 ZXDB ONECUT2 TAOK1 HIPK1

Figure 4: Venn diagram showing unique and common number of genes amongst four shortlisted miRNAs after comparison of their target gene list

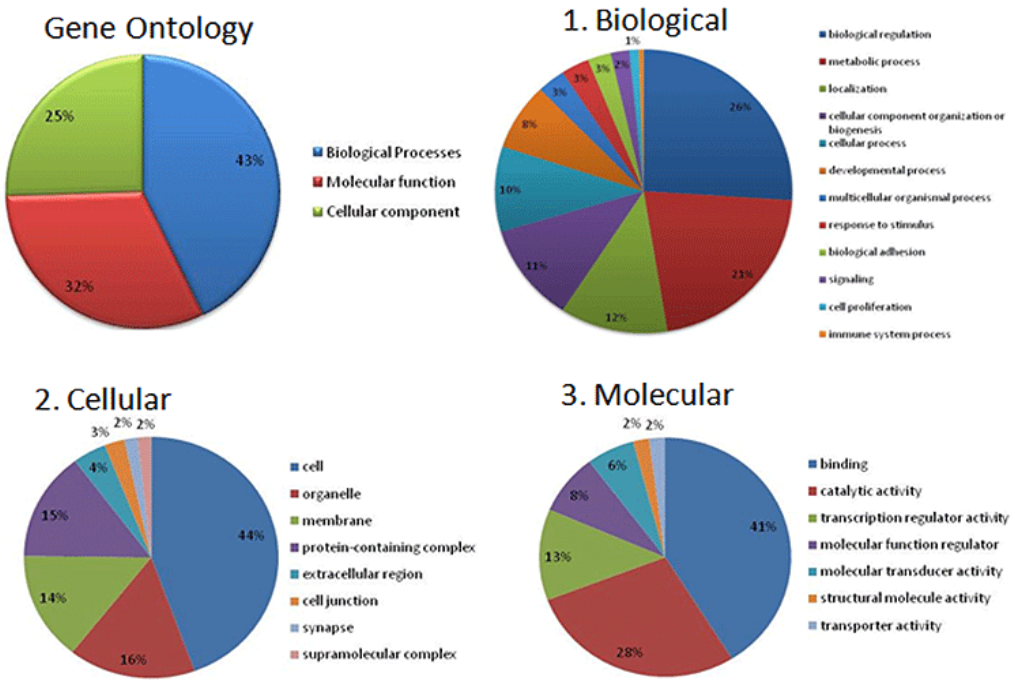


Figure 5: Gene Enrichment analysis: Classification of common target genes from all miRNAs (135) into (1) Biological processes, (2) Cellular component and (3) Molecular functions.

Discussion

Sojourners visiting high altitude face extreme weather conditions including cold and dry winds, intense solar radiations and inevitable hypobaric hypoxia. People staying at altitudes are prone to several HA induced maladies such as Acute Mountain Sickness (AMS), High Altitude Pulmonary Edema (HAPE), High Altitude Cerebral Edema (HACE), sleeplessness and other chronic diseases. A person travelling to HA needs to acclimatize by changes in gene expression in order to minimize the effect of hypoxemia and maintain cellular functions. Despite extensive research on high-altitude adaptations at physiological and genomic levels however a very few have focussed on role of miRNAs in high altitude adaptation. Genetic basis of adaptation of an individual to HA hypoxic environment remains to be completely elucidated.

Venous thrombosis is one of the leading causes of deaths worldwide. DVT and PE are major causes of mortality and morbidity and hence are significant health concerns. Apart from clinical risk factors, there are several genetic/inherited and acquired risk factors playing an important role in VTE predisposition (Souto et al. 2000; Ariens et al. 2002; Zoller et al. 2013). Numerous studies have associated genetic variations in number of genes with VTE etiology however exact genetic and epigenetic mechanisms underlying the disease remains to be fully elucidated (Morange et al. 2015). Thrombotic events have been recorded in Himalayan trekkers more than three decades ago (Dickinson et al. 1983). Thereafter some case reports from high altitude climbers and Himalayan mountaineers have been documented (Cheng et al. 2009, Trogovicky et al. 2005). Other haematological studies have focussed on high altitude induced thrombosis in military personnel (Anand et al. 2005, Nair et al. 2006). It has been well established that various physical and environmental challenges of high altitude including hypoxia, extremely low temperature (Nagelkirk et al. 2012) and enforced inactivity might result in circulatory stasis and other haematological changes such as hypercoagulability leading to thrombosis. However, there are many contradictory reports which do not support the idea that HA exposure contributes to prothrombotic milieu (Toff et al. 2006).

Unlike the previous belief that miRNAs modulate gene expression only by repressing the target mRNA, interestingly some published reports indicate that miRNAs might oscillate between gene repression as well as stimulation in response to varying cellular conditions and cofactors (Vasudevan et al. 2012). Considering the key functions of miRNAs in gene regulation, they are considered as promising future therapeutic targets for various disorders (Li et al. 2010, Rupaimoole et al. 2011). This potential of miRNAs have been widely explored and demonstrated in several studies in the last decade. We performed in-silico computational analysis to evaluate the role of previously reported miRNAs in HA induced VTE prognosis. In the present study a total of 25miRNAs were selected after extensive review of published data. These miRNAs have been shown to be involved in various physiological and biochemical mechanisms such as angiogenesis, platelet activation, platelet production, Tissue Factor (TF) expression and modulation, fibrinogen and collagen production, anti-thrombin production, inhibition of SERPINE1, induction/inhibition of Plasminogen Activator Inhibitor-1 (PAI-1), NFKB signalling and showed differential expression during VTE/DVT. When target genes of each of these miRNAs were subjected to pathway analysis, it revealed that six out of twenty five miRNAs were enriched in both VTE-related pathways (blood coagulation, hemostasis, platelet function and endothelia signalling) as well as hypoxia-related (hypoxia response via Hif activation, angiogenesis, apoptosis and homeostasis) pathways. These miRNAs were hsa-miR-197, hsa-miR-24, hsa-miR-19b, hsa-miR-20a, hsa-miR-106b and hsa-miR-136 table 3. However later when these six miRNAs were subjected to network analysis four out of them miR-20a, hsa-miR-106, hsa-miR-19b and hsa-miR-24 were observed to occupy central position in the network and were found connected to maximum nodes. This indicated that these miRNAs could be playing an important role in high altitude induced VTE patho physiology.

Table 3: Pathway analysis of target genes list of all selected miRNAs: Results of only 8 pathways have been presented here, 4 VTE-linked pathways and 4 hypoxia-linked pathways

S. No.	MicroRNAs	Total genes Hit in Relevant Pathways							
		Pathways relevant to VTE				Pathways relevant to Hypoxia			
		Blood coagulation	Hemostasis	Platelet activation/ signaling and aggregation	Endothelin signaling pathway	Hypoxia response via HIF activation	Angiogenesis	Apoptosis	Homeostasis
1	hsa-miR-126-3p/5p	3	35	17	8	3	19	9	1
2	hsa-miR-197	9	162	79	31	9	49	46	11
3	hsa-miR-21-5p	3	33	17	6	4	19	10	6
4	hsa-miR-150	3	58	21	13	9	26	14	5
5	hsa-miR-24-3p	8	63	28	13	3	28	17	10
6	hsa-miR-145-5p	0	48	17	9	3	25	9	9
7	hsa-miR-29a	1	75	35	10	10	33	20	10

8	hsa-miR-409-3p	4	37	14	7	5	15	8	4
9	hsa-miR-18a	3	53	12	11	6	21	8	2
10	hsa-miR-19b	7	89	38	26	7	37	27	8
11	hsa-miR-20a	10	211	95	30	18	70	49	22
12	hsa-miR-106b	6	96	43	11	8	35	25	11
13	hsa-miR-421	2	52	19	7	4	18	11	8
14	hsa-miR-30c-5p	3	89	37	10	3	30	18	8
15	hsa-miR-301a-3p	3	66	27	14	5	33	25	9
16	hsa-miR-335-5p	3	46	21	8	5	18	9	9
17	hsa-miR-10b-5p	2	16	7	2	4	9	10	3
18	hsa-miR-320a/b	3	71	30	11	7	29	16	6
19	hsa-miR-424-5p	3	110	47	23	13	54	24	17
20	hsa-miR-423-5p	2	46	22	9	3	13	3	4
21	hsa-miR-103a-3p	2	93	31	14	7	35	17	17
22	hsa-miR-191-5p	3	43	15	6	3	17	6	8
23	hsa-mir-199b-3p	4	93	55	20	6	35	25	10
24	hsa-miR-136	6	128	53	22	9	40	34	13
25	hsa-miR-26a	2	55	6	12	7	19	15	6

Second priority list of miRNAs included hsa-miR-29a, hsa-miR-320a, hsa-miR-424 and hsa-miR-103a table 3. These miRNAs also target large number of genes of VTE related pathways (except blood coagulation pathway directly) and hypoxia-related pathways. Interestingly, in one of our previous publications on circulating miRNAs in relation to venous thrombosis (Srivastava et al. 2018) also indicated significance of hsa-miR-320 and hsa-miR-103 in VTE patho physiology.

Present in-silico analysis gives a panel of four miRNAs; miR-20a, hsa-miR-106, hsa-miR-19b and hsa-miR-24, which have been validated for their role in pathogenesis of VTE previously table 1, and we now suggest that they could be playing a crucial role in HA induced VTE as well. We also used Venn diagram to identify the common target genes amongst the four top listed miRNAs figure 4. One hundred thirty five genes were found to be commonly targeted by all four miRNAs. These genes are listed in figure 4. Ontological analysis of these genes revealed that they belonged to various biological processes including response to stimulus, adhesion and signalling.

The data presented in the present study is the first step in identifying miRNAs that having significant role in HA-VTE patho physiology. Furthermore, extensive computational study and experimental validation of four miRNAs shortlisted here may be carried out for better understanding the mechanism of miRNA based gene regulation during VTE. In future, these miRNAs may also prove to have therapeutic relevance for VTE treatment.

Conclusion

As global popularity to visit high altitude areas continue to increase it is much more needed to spread awareness about HA induced maladies and their early recognition symptoms, preventive measures and appropriate therapy to minimize morbidity and mortality. The excitement of miRNA based research and their potential clinical application is building up in today's world. Our present in-silico study provides novel insights into path physiology of high altitude induced venous thrombosis. We have identified condensed panel of four miRNAs and their commonly targeted genes that could be playing a crucial role in determining the etiology of high altitude induced venous thrombo-embolism. These miRNAs can be exploited in future for therapeutic applications for treatment of HA-VTE. However, further validation is required to establish the significance of these miRNAs in HA induced VTE pathogenesis and elucidate its mechanism completely.

References

1. Agnelli G, Buller HR, Cohen A, Curto M, Gallus AS, Johnson M, et al. Oral apixaban for the treatment of acute venous thromboembolism. N Engl J Med. 2013;369(9):799-808. doi: 10.1056/NEJMoa1302507
2. Anand AC, Saha A, Seth AK, Chopra GS, Nair V, Sharma V. Symptomatic portal system thrombosis in soldiers due to extended stay at extreme altitude. J Gastroenterol Hepatol. 2005;20(5):777-783.
3. Anand AC, Jha SK, Saha A, Sharma V, Adya CM. Thrombosis as a complication of extended stay at high altitude. Natl Med J India. 2001;14(4):197-201.
4. Ariëns RA, de Lange M, Snieder H, Boothby M, Spector TD, Grant PJ. Activation markers of coagulation and fibrinolysis in twins: heritability of the pre-thrombotic state. Lancet. 2002;359(9307):667-671. doi: 10.1016/S0140-6736(02)07813-3
5. Bao CX, Zhang DX, Wang NN, Zhu XK, Zhao Q, Sun XL. MicroRNA-335-5p suppresses lower extremity deep venous thrombosis by targeted inhibition of PAI-1 via the TLR4 signalling pathway. J Cell Biochem. 2018;119(6):4692-4710. doi: 10.1002/jcb.26647

6. Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell. 2004;116(2):281-297.
7. Bartsch P. How thrombogenic is hypoxia. Journal of the American Medical Association. 2006;295(19):2297-2299.
8. Bauersachs R, Berkowitz SD, Brenner B, Buller HR, Decousus H, Gallus AS, et al. Oral rivaroxaban for symptomatic venous thromboembolism. N Engl J Med. 2010;363(26):2499-2510.
9. Bovill EG, van der Vliet A. Venous valvular stasis-associated hypoxia and thrombosis: what is the link? Annu Rev Physiol. 2011;73:527-545. doi: 10.1146/annurev-physiol-012110-142305
10. Brown JM. Tumor hypoxia in cancer therapy. Methods Enzymol. 2007;435:297-321. doi: 10.1016/S0076-6879(07)35015-5
11. Büller HR, Décousus H, Grosso MA, Mercuri M, Middeldorp S, Prins MH, et al. Edoxaban versus warfarin for the treatment of symptomatic venous thromboembolism. N Engl J Med. 2013;369(15):1406-1415. doi: 10.1056/NEJMoa1306638
12. Carino A, De Rosa S, Sorrentino S, Polimeni A, Sabatino J, Caiazzo G, et al. Modulation of circulating microRNAs levels during the switch from clopidogrel to ticagrelor. Biomed Res Int. 2016;3968206:5.
13. Cheng S, Chng SM, Singh R. Cerebral venous infarction during a high altitude expedition. Singapore Med J. 2009;50(8):e306-e308.
14. Cheng Y, Zhang C. MicroRNA-21 in cardiovascular disease. J Cardiovasc Transl Res. 2010;3(3):251-255. doi: 10.1007/s12265-010-9169-7
15. Corsten MF, Dennert R, Jochems S, Kuznetsova T, Devaux Y, Hofstra L, et al. Circulating microRNA-208b and microRNA-499 reflect myocardial damage in cardiovascular disease. Circ Cardiovasc Genet. 2010;3(6):499-506. doi: 10.1161/CIRCGENETICS.110.957415
16. Fabregat A, Jupe S, Matthews L, Sidiropoulos K, Gillespie M, Garapati P, et al. The Reactome pathway knowledgebase. Nucleic Acids Res. 2018;46(D1):D649-D655. DOI: 10.1093/nar/gkx1132
17. Alessandra YD, Devanna P, Limana F, Straino S, Di CA, Brambilla PG, et al. circulating microRNAs are new and sensitive biomarkers of myocardial infarction. Eur Heart J. 2010;31(22):2765-2773.
18. de Boer HC, van Solingen C, Prins J, Duijs JM, Huisman MV, Rabelink TJ, et al. Aspirin treatment hampers the use of plasma microRNA-126 as a biomarker for the progression of vascular disease. Eur Heart J. 2013;34(44):3451-3457. doi: 10.1093/eurheartj/eht007
19. Dickinson J, Heath D, Gosney J, Williams D. Altitude- related deaths in seven trekkers in the Himalayas. Thorax. 1983;38(9):646-656.
20. Fabregat A, Jupe S, Matthews L, Sidiropoulos K, Gillespie M, Garapati P, et al. The Reactome Pathway Knowledgebase. Nucleic Acids Res. 2018;46(D1):D649-D655. doi: 10.1093/nar/gkx1132
21. Fan Y, Siklenka K, Arora SK, Ribeiro P, Kimmins S, Xia J. miRNet - dissecting miRNA-target interactions and functional associations through network-based visual analysis. Nucleic Acids Res. 2016;44(W1):W135-W141. doi: 10.1093/nar/gkw288
22. Fan Y, Xia J. miRNet: functional analysis and visual exploration of miRNA-target interactions in a network context. Methods Mol Biol. 2018;1819:215-233. doi : 10.1007/978-1-4939-8618-7_10
23. Fish RJ, Neerman-Arbez M. Fibrinogen gene regulation. Thromb Haemost. 2012;108(3):419-426. doi: 10.1160/TH12-04-0273
24. Fort A, Borel C, Migliavacca E, Antonarakis SE, Fish RJ, Neerman-Arbez M. Regulation of fibrinogen production by microRNAs. Blood. 2010;116(14):2608-2615. doi: 10.1182/blood-2010-02-268011
25. Greco S, Zaccagnini G, Voellenkle C, Martelli F. microRNAs in ischaemic cardiovascular diseases. Eur Heart J Suppl. 2016;18(Suppl E):E31-E36. doi: 10.1093/eurheartj/suw012
26. Pulivarthi S, Gurram MK. Effectiveness of d-dimer as a screening test for venous thromboembolism: an update. N Am J Med Sci. 2014;6(10):491-499. doi: 10.4103/1947-2714.143278
27. Hatziapostolou M, Polytarchou C, Aggelidou E, Drakaki A, Poultsides GA, Jaeger SA, et al. An HNF4alpha-miRNA inflammatory feedback circuit regulates hepatocellular oncogenesis. Cell. 2011;147(6):1233-1247. doi: 10.1016/j.cell.2011.10.043
28. Heit JA. The epidemiology of venous thromboembolism in the community. Arterioscler Thromb Vasc Biol. 2008;28(3):370-372.
29. Huaiyu Mi, Xiaosong Huang, Anushya Muruganujan, Haiming Tang, Caitlin Mills, Diane Kang, et al. PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. Nucl Acids Res. 2017;45(Database issue):D183-D189.
30. Karolina DS, Tavintharan S, Armugam A, Sepramaniam S, Pek SL, Wong MT, et al. Circulating miRNA profiles in patients with metabolic syndrome. J Clin Endocrinol Metab. 2012;97(12):E2271-E2276.
31. Khalil KF, Saeed W. Pulmonary embolism in soldiers serving at high altitude. J Coll Physicians Surg Pak. 2010;20(7):468-471. doi: 07.2010/JCPSP.468471
32. Koh MY, Powis G. Passing the baton: the HIF switch. Trends Biochem Sci. 2012;37(9):364-372.
33. Kulshreshtha R, Ferracin M, Wojcik SE, Garzon R, Alder H, Agosto-Perez FJ, et al. A microRNA signature of hypoxia. Mol Cell Biol. 2007;27(5):1859-1867. doi: 10.1128/MCB.01395-06
34. Li M, Li J, Ding X, He M, Cheng SY. Micro RNA and cancer. The AAPS Journal. 2010;12(3):309-317.
35. Li Zi, Jiangdong Ni. Role of microRNA-26a in the diagnosis of lower extremity deep vein thrombosis in patients with bone trauma. Exp Ther Med. 2017;14(5):5069-5074.
36. Lu-Jia Chen, Lian Yang, Xing Cheng, Yin-Kai Xue, Li-Bo Chen. Over expression of miR-24 Is Involved in the Formation of Hypo coagulation State after Severe Trauma by Inhibiting the Synthesis of Coagulation Factor X. Dis Markers. 2017:3649693. doi: 10.1155/2017/3649693
37. Marchand A, Proust C, Morange PE, Lompre AM, Tregouet DA. miR-421 and miR-30c inhibit SERPINE 1 gene expression in human endothelial cells. PLoS One. 2012;7(8):e44532. doi: 10.1371/journal.pone.0044532
38. Mayr M, Zampetaki A, Willeit P, Willeit J, Kiechl S. MicroRNAs within the continuum of post genomics biomarker discovery. Arterioscler Thromb Vasc Biol. 2013;33(2):206-214.
39. Mi H, Dong Q, Muruganujan A, Gaudet P, Lewis S, Thomas PD. PANTHER version 7: improved phylogenetic trees, orthologs and collaboration with the Gene Ontology Consortium. Nucleic Acids Res. 2010;38(DI):D204-D210. doi: 10.1093/nar/gkp1019
40. Morange PE, Suchon P, Treqouet DA. Genetics of Venous thrombosis: update in 2015. Thromb Haemost. 2015;114(5):910-919.
41. Nagelkirk PR, Hogan KB, Hoare JM. Ambient temperature affects thrombotic potential at rest and following exercise. Thromb Res. 2012;130(2):248-252. doi: 10.1016/j.thromres.2011.10.015

42. Nair V, Sharma A, Das SR, Mishra DK, Kotwal J, Agarwal MB. Thrombotic complications in young adults at high altitude areas: An Indian experience. Blood. 2006;108(11):4108.
43. Noman MZ, Janji B, Berchem G and Chouaib S. miR-210 and hypoxic microvesicles: two critical components of hypoxia involved in the regulation of killer cells function. Cancer Lett. 2016;380(1):257-262.
44. Patel N, Tahara SM, Malik P, Kalra VK. Involvement of miR-30c and miR-301a in immediate induction of plasminogen activator inhibitor-1 by placental growth factor in human pulmonary endothelial cells. Biochem J. 2011;434(3):473-482. doi: 10.1042/BJ20101585
45. Peterson SM, Thompson JA, Ufkin ML, Sathyanarayana P, Liaw L, Congdon CB. Common features of microRNA target prediction tools. Front Genet. 2014;5:23. doi: 10.3389/fgene.2014.00023
46. Pichler Hefti J, Risch L, Hefti U, Scharrer I, Risch G, Merz TM, et al. Changes of coagulation parameters during high altitude expedition. Swiss Medical Weekly. 2010;140(7-8):111-117. doi: smw-12910
47. Rathi KR, Uppal V, Bewal NM, Sen D, Khanna A. D-dimer in the diagnostic workup of suspected pulmonary thrombo-embolism at high altitude. Med J Armed Forces India. 2012;68(2):142-144.
48. Rupaimoole R, Han HD, Lopez-Berestein G, Sood AK. MicroRNA therapeutics: principles, expectations, and challenges. Chinese Journal of Cancer. 2011;30(6):368-370.
49. Sahu A, Jha PK, Prabhakar A, Singh HD, Gupta N, Chatterjee T, et al. MicroRNA-145 Impedes Thrombus Formation via Targeting Tissue Factor in Venous Thrombosis. E Bio Medicine. 2017;26:175-186.
50. Shishir K. High altitude induced deep venous thrombosis: A study of 28 cases. Indian Journal of Surgery. 2006;68(2):84-88.
51. Souto JC, Almasy L, Borrell M, Blanco-Vaca F, Mateo J, Soria JM, et al. Genetic susceptibility to thrombosis and its relationship to physiological risk factors: the GAIT study. Genetic Analysis of Idiopathic Thrombophilia. Am J Hum Genet. 2000;67(6):1452-1459.
52. Srivastava S, Garg I, Ganju L, Kumar B. Prediction of circulating miRNAs and their target genes involved in venous thrombo-embolism (VTE) pathogenesis: A computational approach. J Res Rep Genet. 2018;2(3):10-20.
53. Starikova I, Jamaly S, Sorrentino A, Blondal T, Latysheva N, Sovershaev M, et al. Differential expression of plasma miRNAs in patients with unprovoked venous thromboembolism and healthy control individuals. Thromb Res. 2015;136(3):566-572. doi: 10.1016/j.thromres.2015.07.005
54. Teruel R, Corral J, Perez-Andreu V, Martinez-Martinez I, Vicente V, Martinez C. Potential role of miRNAs in developmental haemostasis. PLoS ONE. 2011;6(3):e17648.
55. Teruel R, Perez-Sanchez C, Corral J, Herranz MT, Perez Andreu V, Saiz E, et al. Identification of miRNAs as potential modulators of tissue factor expression in patients with systemic lupus erythematosus and antiphospholipid syndrome. J Thromb Haemost. 2011;9(10):1985-1992.
56. Toff WD, Jones CI, Ford I, Pearse RJ, Watson HG, Watt SJ, et al. Effect of hypobaric hypoxia, simulating conditions during long-haul air travel, on coagulation, fibrinolysis, platelet function and endothelial activation. JAMA. 2006;295(19):2251-2261. doi: 10.1001/jama.295.19.2251
57. Tokar T, Pastrello C, Rossos AEM, Abovsky M, Hauschild AC, Tsay M, et al. mirDIP 4.1-integrative database of human microRNA target predictions. Nucleic Acids Res. 2018;46(D1):D360-D370.
58. Torgovicky R, Azaria B, Grossman A, Eliyahu U , Goldstein L. Sinus vein thrombosis following exposure to simulated high altitude. Aviat Space Environ Med. 2005;76(2):144-146.
59. Van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, et al. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci. 2008;105(35):13027-13032. doi: 10.1073/pnas.0805038105
60. Vasudevan S. Post-transcriptional upregulation by microRNAs. Wiley Interdisciplinary Reviews: RNA. 2012;3(3):311-330. doi: 10.1002/wrna.121
61. Vij AG. Effect of prolonged stay at high altitude on platelet aggregation and fibrinogen levels. Platelets. 2009;20(6):421-427. doi: 10.1080/09537100903116516
62. Vlachos IS, Paraskevopoulou MD, Karagkouni D, Georgakilas G, Vergoulis T, Kanellos I, et al. DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions. Nucleic Acids Res. 2015;43(DI):D153-D159. doi: 10.1093/nar/gku1215
63. Wang F, Long G, Zhao C, Li H, Chaugai S, Wang Y, et al. Atherosclerosis related circulating miRNAs as novel and sensitive predictors for acute myocardial infarction. PLoS One. 2014;9(9):e105734.
64. Wang K, Yuan Y, Cho JH, McClarty S, Baxter D, Galas DJ. Comparing the MicroRNA spectrum between serum and plasma. PLoS One. 2012;7(7):e41561.
65. Wang X, Sundquist K, Elf JL, Strandberg K, Svensson PJ, Hedelius A, et al. Diagnostic potential of plasma microRNA signatures in patients with deep-vein thrombosis. Thromb Haemost. 2016;116(2):328-336. doi: 10.1160/TH16-01-0071
66. Willeit P, Zampetaki A, Dudek K, Kaudewitz D, King A, Kirkby NS, et al. Circulating microRNAs as novel biomarkers for platelet activation. Circ Res. 2013;112(4):595-600. doi: 10.1161/CIRCRESAHA.111.300539
67. Wong N, Wang X. miRDB: an online resource for microRNA target prediction and functional annotations. Nucleic Acids Research. 2015;43(D1):D146-D152. doi: 10.1093/nar/gku1104
68. Yu G, Li H, Wang X, Wu T, Zhu J, Huang S, et al. MicroRNA-19a targets tissue factor to inhibit colon cancer cells migration and invasion. Mol Cell Biochem. 2013;380(1-2):239-247. doi: 10.1007/s11010-013-1679-6
69. Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, et al. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. Circ Res. 2010;107(6):810-817. doi: 10.1161/CIRCRESAHA.110.226357
70. Zampetaki A, Willeit P, Tilling L, Drozdov I, Prokopi M, Renard JM, et al. Prospective study on circulating microRNAs and risk of myocardial infarction. J Am Coll Cardiol. 2012;60(4):290-239.
71. Zhang X, Yu H, Lou JR, Zheng J, Zhu H, Popescu NI, et al. MicroRNA-19 (miR-19) regulates tissue factor expression in breast cancer cells. J Biol Chem. 2011;286(2):1429-1435.
72. Zöller B, Ohlsson H, Sundquist J, Sundquist K. Familial risk of venous thrombo-embolism in first-, second- and third-degree relatives: a nationwide family study in Sweden. Thromb Haemost. 2013;109(3):458-463. doi: 10.1160/TH12-10-0743