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Research Paper

MicroRNA-145 Impedes Thrombus Formation *via* Targeting Tissue Factor in Venous Thrombosis

Anita Sahu [a], Prabhash Kumar Jha [a], Amit Prabhakar [a], Heisnam Dinesh Singh [b], Neha Gupta [a], Tathagata Chatterjee [c], Tarun Tyagi [a], Swati Sharma [a], Babita Kumari [a], Somnath Singh [a], Velu Nair [d], Shailendra Goel [b], Mohammad Zahid Ashraf [a,e,*]

[a] *Defence Institute of Physiology and Allied Sciences, DRDO, Delhi, India*
[b] *Department of Botany, University of Delhi, Delhi, India*
[c] *Army Hospital (Research and Referral), New Delhi, India*
[d] *Armed Forces Medical College, Pune, India*
[e] *Department of Biotechnology, Jamia Millia Islamia, New Delhi, India*

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ABSTRACT

Venous thromboembolism (VTE), the third leading cardiovascular complication, requires more understanding at molecular levels. Here, we have identified miR-145 as a key molecule for regulating thrombus formation in venous thrombosis (VT) employing network based bioinformatics approach and *in vivo* experiments. Levels of miR-145 showed an inverse correlation with thrombus load determined by coagulation variables. MiRNA target prediction tools and *in vitro* study identified tissue factor (TF) as a target gene for miR-145. The restoration of miR-145 levels in thrombotic animals *via in vivo* miR-145 mimic delivery resulted in decreased TF level and activity, accompanied by reduced thrombogenesis. MiR-145 levels were also reduced in VT patients and correlated with increased TF levels in patients, thereby, confirming our preclinical findings. Our study identifies a previously undescribed role of miRNA in VT by regulating TF expression. Therefore, restoration of miR-145 levels may serve as a promising therapeutic strategy for management of VT.

1. Introduction

Venous thromboembolism (VTE) is a leading cardiovascular complication affecting 100–200 individuals in a population of 100,000 per year and emerging as a third largest cardiovascular disease (Malone and Agutter, 2006). A combination of genetically manipulated animal models and human epidemiological data has revealed that a variety of genetic and acquired risk factors are associated with VTE, which contribute to its multifactorial complex nature (Diaz et al., 2012; Heit et al., 2002). These complexities might be the reason for the continuation of antithrombotic drugs for decades in spite of the risk of bleeding associated with their use. Despite the extensive clinical evidence about VTE, better molecular understanding is required to explore further and identify specific therapeutic targets.

MiRNAs are non-coding RNAs modulating gene expression, either through mRNA degradation or translational repression of multitude of targets (Bartel, 2004). Each miRNA has the potential to simultaneously target multiple mRNAs, and repress genes found in similar pathways to alter biological networks (Bartel, 2009; Selbach et al., 2008). Till date, various experimental reports have highlighted the functional role of miRNAs in cardiovascular biology, physiology, and diseases (Small et al., 2010). Several miRNAs including miR-155, miR-126, miR-21 and miR-146 have been reported to regulate pathogenic signaling in the development and progression of vascular inflammation, neointimal lesion formation, atherosclerosis and coronary artery disease (Ji et al., 2007; Liu et al., 2009; Weber et al., 2010). However, to our knowledge, the precise role of miRNAs in the etiopathology of VT has not been shown yet. Keeping the essence of all these facts, the present study aimed to identify the miRNA signatures that could be of paramount importance in the understanding of the pathogenesis of thrombus formation in the venous milieu.

In the present study, using both network based bioinformatics approach and *in vivo* animal model system, we explored the possible role of miRNAs in VT. Importantly, our results demonstrated that the reduced levels of miR-145 were associated with enhanced thrombus formation. Furthermore, we also provided the experimental evidence for identifying tissue factor (TF), as a direct target for miR-145 with binding sites at the 3′UTR of the gene. The restoration of miR-145 levels *via in vivo* miRNA mimic delivery inhibited TF expression and attenuated thrombus formation in the animal model of VT, thereby indicating its

* Corresponding author at: Genomics Division, Defence Institute of Physiology and Allied Sciences, Lucknow Road, Timarpur, Delhi 110 054, India.
E-mail address: zashraf@jmi.ac.in (M.Z. Ashraf).

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antithrombotic potential. Nonetheless, our preclinical findings were also validated in human VT patients, supporting the potential translational significance of miR-145 against VT.

2. Experimental Procedures

2.1. Screening of miRNAs From Disease-miRNAs Network by Network Topology Analysis

We exploited the curated list of miRNAs with obvious importance in related vascular diseases (flow chart shown in Fig. S1A) like atherosclerosis, acute coronary syndrome, myocardial infarction as well as pathophysiological states including inflammation, endothelial cell activation, ischemia, and neoplasm. Briefly, validated miRNAs related to these diseases and pathophysiological states were obtained from miRWalk database (list shown in Supplementary excel file 1). A network of diseases and miRNAs was created. Further, the network was analyzed based on complex centrality scoring algorithms (Fig. S1B-S1D, details in Supplementary excel file 2) and a list of candidate miRNAs with higher scores was generated. The detailed method applied for network based bioinformatics is further described in Supplementary methods.

2.2. Animal Studies

All experiments were conducted in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (IAEC-03/DIPAS/2011), Government of India. Age matched 250–300 g Sprague-Dawley rats were used. Stasis induced venous thrombosis animal model was implemented as previously described (Henke et al., 2004; Downing et al., 1998). Briefly, rats were anesthetised with ketamine (100 mg/kg), xylazine (20 mg/kg) and placed in the supine position. Following a midline laparotomy, the intestines were exteriorized and placed to the left of the animal and the inferior vena cava (IVC) was carefully separated from the surrounding tissues and then ligated just below the renal veins along with ligation of side branches. After 6 h, 12 h and 48 h of ligation, rats were euthanized, the IVC was carefully dissected. Thrombus was extracted, weight of the thrombus was measured in milligrams and length was determined in millimeters. The IVC with thrombus was fixed immediately in formalin and later stained with hematoxylin and eosin.

2.3. RNA Isolation and Quantitative RT-PCR Analysis

Total RNA was extracted from IVC tissue using TRIzol reagent (Sigma) according to the manufacturer's suggested protocol. Isolation of intimal and media RNA from IVC was modified from a previous study (Sun et al., 2012). Briefly, IVC was cut out and transferred to a dish containing ice-cold phosphate buffer saline (PBS). The tip of an insulin syringe needle was carefully inserted into one end of the IVC to facilitate a quick flush of QIAzol lysis buffer, and intima eluate was collected. The IVC leftover (media + adventitia) was washed once with PBS and snap-frozen in liquid nitrogen, for storage until total RNA extraction by TRIzol. RNA from platelets and PBMCs were isolated as previously described (Konieczna et al., 2015; Tyagi et al., 2014). The detailed method is further described in Supplementary methods. MiRNA qRT-PCR was performed with Stem-loop qRT-PCR method (Chen et al., 2005). Briefly, 500 ng of total RNA was reverse-transcribed *via* SuperScript-III reverse transcriptase (Invitrogen) using miRNA specific RT primers. For mRNA qRT-PCR cDNA was synthesized from 1 μg of total RNA using the Quantitect Reverse Transcription Kit (Qiagen) in accordance with the manufacturer's instructions. QRT-PCR was performed using a CFX 96 connect real-time PCR system (BioRad). Expression was normalized to the housekeeping gene. Small RNA housekeeping control was 5S ribosomal RNA for miRNA and 18S for mRNA. Primer sequences are shown in Tables S2 and S3.

2.4. Analysis of Potential Targets of Differentially Expressed miRNAs

Potential targets of miRNAs were identified using online available miRNA prediction tool miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) (Dweep et al., 2012). For accurate prediction, targets predicted by at least three tools and conserved across rat, mouse, and human were selected (Supplementary excel file 3). Selected target genes were analyzed by GeneCodis (Tabas-Madrid et al., 2012; Carmona-Saez et al., 2007; Nogales-Cadenas et al., 2009) to obtain enriched pathways with adjusted $p < 0.05$ as the cut-off.

2.5. In vitro Luciferase Reporter Assay

A partial TF 3′UTR sequence of 434 base pairs containing miR-145 target sites was cloned into psiCHECK-2 Vector (Promega) downstream of Renilla luciferase gene using the *Xho*I and *Not*I restriction sites. Mutagenesis of miR-145 target sites was performed by PCR splicing method, and mutated 3′UTR sequences were also cloned into psiCHECK-2 Vector. Primers used for plasmid construction (psiCHECK-2 and TF 3′UTR or mutated TF 3′UTR) are listed in table S4. HEK293 cells were seeded in 24-well plates in DMEM supplemented with 10% FBS and maintained in 5% CO_2 incubator at 37 °C. The transfection was done after 24 h using Lipofectamine 3000 (Invitrogen) as per manufacturer's protocol. Cells were transfected with 100 ng of psiCHECK-2 constructs and 40 nM of Pre-miR™ miRNA precursor molecules-negative Control # 1 (non-specific/scr. mimic) and Rno-miR-145 Pre-miR™ miRNA precursor (miR-145 mimic, Ambion). Cells were harvested 24 h post transfection. Luciferase assay was performed by using Dual Luciferase Reporter Assay System (Promega) following the manufacturer's protocol. Firefly luciferase activity was normalized to Renilla luciferase activity and expressed as relative light units (RLU). The relative luciferase activity was reported as the fold change between scr. mimic and miR-145 mimic transfected cells.

2.6. In vivo miRNA Delivery

For *in vivo* application, miRNA mimics and inhibitors (*in vivo* grade, Invitrogen) were suspended in invivofectamine (Invitrogen) according to manufacturer's instructions. Invivofectamine 2.0 was used to form nanoparticles suitable for *in vivo* applications. mirVana miRNA mimics and inhibitors (Life Technologies) were complexed with Invivofectamine 2.0 reagent. Each animal ($n = 5$ per group) was administered with 200 μl mixtures containing scr. mimic/inhibitor or miR-145 mimic/inhibitor (1 nmol–10 nmol) by tail vein injection before 1 h of IVC ligation, as described in a previous study (Sun et al., 2012).

2.7. Histological Examination

After extraction IVC containing thrombus, liver, heart and kidney tissue were collected in 10% buffered formalin and embedded in paraffin. Serial cross sections (5 μm) of the IVC with thrombus were cut to analyze thrombus formation. Tissue sections were stained with hematoxylin and eosin following standard procedures. Liver, heart, and kidney tissue sections were analyzed to search for signs of any fibrosis, inflammation or toxicity. All histological images were acquired using an inverted microscope (Motic) and analyzed by Motic Images 2.0 software.

2.8. Western Blotting

Proteins from IVC tissues were extracted with RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% NP40, 0.25% Na-deoxycholate and 0.1% SDS) containing complete protease inhibitor cocktail. 30–50 μg of protein was loaded on SDS–polyacrylamide gel electrophoresis gel for separation, which was followed by blotting of protein on polyvinylidene fluoride membrane in semi-dry trans-blot

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