Relative Expression of PBMC MicroRNA-133a Analysis in Patients Receiving Warfarin After Mechanical Heart Valve Replacement

Hamid Kabiri Rad 1, Mahta Mazaheri 2*, and Ali Dehghani Firozabadi 3

1. International Campus, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
2. Department of Medical Genetics, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
3. Yazd Cardiovascular Research Center, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

Abstract

Background: MicroRNAs (miRNAs) are implicated in various biological processes including anticoagulation. However, the modulation of miRNA by pharmacological intervention such as warfarin treatment in patients receiving warfarin has not been disclosed yet. The aim of this study work was to assess the effect of warfarin drug on expression level of mir-133a-3p in patients with mechanical heart valve replacement.

Methods: In this research, the expression level of miRNA-133a-3p was analyzed in Peripheral Blood Mononuclear Cells (PBMCs) from mechanical valve replacement patients who had received warfarin for at least 3 months continuously. Quantitative RT-PCR method was used for this assay.

Results: Our findings indicated a significant difference between the rate of miR-133a-3p expression in individuals receiving warfarin and the control group (p<0.01). There was also a statistically significant difference in miR-133a-3p expression in patients with different ages (p<0.05) suggesting that the rate of miR-133a-3p expression in persons receiving warfarin is related to age. However, other variables like warfarin dose, International Normalized Ratio (INR), gender, and Body Mass Index (BMI) were not significantly effective on the miR-133a-3p expression rate in individuals receiving warfarin.

Conclusion: Based on our results, it can be concluded that miR-133a-3p is involved in the coagulation pathway. The recent result indicates that warfarin affects the expression of miR-133a. This expression may be potentially important for treatment by anti-coagulants. Awareness of the time course of miRNA expression profile can improve efficiency of response to warfarin.

Keywords: Anticoagulation, MicroRNAs, Quantitative reverse transcription-PCR, Warfarin

Introduction

The cardiovascular diseases are the most common causes of mortality worldwide. These artery diseases including coronary, cardiac ischemia, valvular failure, congenital heart defects, and heart diseases are induced by other diseases such as diabetes. Valvular disorder is one of the heart diseases which may lead to cardiac dysfunctions and, ultimately, may result in heart transplantation. One therapeutic strategy in these patients is heart valve repair and in the case of inefficacy of the repair, valve replacement including the biologic and mechanical valves should be considered. One of the most important challenges for this valve replacement is the risk of thrombosis. Patients with valve replacement of the mechanical type need to receive anti-coagulation drugs like warfarin for a lifetime due to the high risk of thrombosis 1,2. The use of warfarin significantly reduces the thrombolytic damages 3,4.

A vitamin K antagonist called Warfarin reduces blood clotting through disturbance in vitamin K cycle and also, it interrupts the synthesis of the coagulation factors IX, VII, II, and X through inhibiting V K O R C I 5,6. Since the administration of warfarin is associated with a high risk of hemorrhage, its function must be repeatedly assessed via the laboratory test of International Normalized Ratio (INR). Increase of individuals’ hypersensitivity to warfarin consumers in different communities promotes the risk of hemorrhage and the possible subsequent death in some cases. Moreover, it has been observed that dosage adjustment basis on INR has not been successful in about 55% of cases 7.
initiation of the primary dose of warfarin can be estimated based on genetic testing which leads to reduction in the time required for achieving the desirable INR. This time requirement can decrease the risk of creation of high INR (associated with haemorrhage) or low INR (associated with thrombosis). Due to genetic differences among individual patients, the prescription and administration of a specific dose of warfarin for all patients is not possible. So far, most studies have focused on factors such as genotype (genetic variant) and demographic differences (such as age, gender and weight) which may potentially affect the warfarin dosage. However, a few studies have shown the role of regulatory factors of genes involved in the metabolism of this drug.

MiRNAs are a group of small single strand non-coding RNAs which interrupt the expression of genes in the post-transcription level.

The essential role of miRNAs in various biological processes such as inflammation, DNA repair, response to oxidative stress, apoptosis, cancer, and cellular growth and the post-transcription regulation of gene expression has been confirmed. The inhibitory role of miR-133a in the pathogenesis of Gall Bladder Carcinoma (GBC) via gene targeting and miR-133a may exert anti-tumor effects on a variety of cancers. The role of miR-133a-3p in regulating genes such as CYP2C9 and VKORC1 in different patients has been investigated. Nonetheless, the effect of warfarin on the expression rate of miR133a-3p has not been approved. Consequently, this study tried to compare the rate of relative expression of miR-133a-3p in mechanical valve replacement patients receiving warfarin to patients in control group and also to investigate its correlation with the received warfarin dose.

**Materials and Methods**

In this experimental research, the participants of this study were mechanical valve replacement patients referred to heart center of Afshar hospital in Yazd province (aged 45+ years) who had received warfarin for at least 3 months with no change in warfarin dose over the last month with an INR limit of 2-3.5. The case (12 patients) and control (10 patients) groups were matched by sex and age (Table 1). Patients’ demographic information was collected by interview and through their medical records. Having obtained informed consent, all the patients voluntarily participated in the study with no treatment limitations for the patients during the completion of the study.

**Separation of peripheral blood mononuclear cells from blood samples by density gradient centrifugation**

Separation of PBMCs from whole blood is usually accomplished through density gradient centrifugation using Ficoll. After the centrifugation step, Ficoll separates layers of blood, with lymphocytes and monocytes under a layer of plasma. Peripheral blood was collected in BD Vacutainer spray-coated K2EDTA Tubes. With-

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Warfarin treated group (n=12)</th>
<th>Non treated group (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years) mean±SD</td>
<td>55.2±8</td>
<td>54.16±7.3</td>
</tr>
<tr>
<td>Height (cm) mean±SD</td>
<td>155.13±24.56</td>
<td>169.16±6.64</td>
</tr>
<tr>
<td>Weight (kg) mean±SD</td>
<td>69.16±6.1</td>
<td>68.66±5.2</td>
</tr>
<tr>
<td>Body mass index, kg/m² mean±SD</td>
<td>24.27±1.59</td>
<td>24.1±2.6</td>
</tr>
<tr>
<td>INR mean±SD</td>
<td>2.3±0.39</td>
<td></td>
</tr>
<tr>
<td>Male/female (n/n)</td>
<td>8/4</td>
<td>4/6</td>
</tr>
<tr>
<td>Warfarin (mg/per week) mean±SD</td>
<td>34.16±10.53</td>
<td></td>
</tr>
<tr>
<td>Aspirin, n%</td>
<td>58.3</td>
<td></td>
</tr>
<tr>
<td>Statin, n%</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>16.6</td>
<td></td>
</tr>
</tbody>
</table>

in 2 hr of blood aspiration, PBMCs were isolated by using Ficoll gradient centrifugation. Buffy coat layer containing Peripheral Blood Mononuclear Cells (PBMCs) was stored at -80°C until use.

**RNA extraction and purification**

Blood samples of patient were used for RNA manipulation. Firstly, for miRNA133a-3p (5'-UUUGGU-CCCUUCAAACCGUG-3') detection, blood samples were collected in EDTA-K2 tubes and incubated at room temperature during 1 hr. A two-step centrifugation (4°C at 820×g for 10 min, then 4°C at 16000×g for 10 min) was done and then the supernatant phase was transferred to RNase/DNase-free tubes and stored at -80°C. Total RNA was extracted by plasma samples using the column RNA isolation Kit (Denazist, Iran) according to the manufacturer’s instructions. The purity and concentration of RNA were determined by OD 260/280 readings using a Nanodrop spectrophotometer (Nanodrop, Thermo Scientific, Germany). RNA integrity was determined by capillary electrophoresis (Biorad, Germany). The quantity and quality of RNA was assayed by using nanodrop spectrophotometer (Nanodrop, Thermo Scientific, Germany) and 2% agarose gel electrophoresis, respectively. In the case of the presence of two 18S and 28S ribosomal RNA bands, the quality of the extracted RNA was approved. The analysis of miRNA-133a expression in PBMCs was performed by miRNA synthesis kit using the Universal RT miRNA PCR, Polyadenylation and cDNA synthesis kit (Pars Genome Co., Iran). cDNA was diluted 5× and assayed in 20 μl PCRs according to the protocol for RT miRNA PCR.

**Quantitative real time PCR analysis**

Expression analysis of miRNA-133a was carried out in an Applied Biosystem Step One plus Real-Time PCR System (ABI, USA). PCR MasterMix for Syber Green Assays (Hot Tag EvaGreen, ROX, GeneAll, South Korea) was used to monitor light cycles of PCR, according to the manufacturer’s protocol. The amplification condition was 95°C for 5 min, 40 cycles at 95°C for 15 s and 60°C for 1 min. Melting curves analysis was performed after each reaction to exclude non specific amplifications. The optimal baseline and threshold

---

**Table 1. Demographic information of the patients**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Warfarin treated group (n=12)</th>
<th>Non treated group (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years) mean±SD</td>
<td>55.2±8</td>
<td>54.16±7.3</td>
</tr>
<tr>
<td>Height (cm) mean±SD</td>
<td>155.13±24.56</td>
<td>169.16±6.64</td>
</tr>
<tr>
<td>Weight (kg) mean±SD</td>
<td>69.16±6.1</td>
<td>68.66±5.2</td>
</tr>
<tr>
<td>Body mass index, kg/m² mean±SD</td>
<td>24.27±1.59</td>
<td>24.1±2.6</td>
</tr>
<tr>
<td>INR mean±SD</td>
<td>2.3±0.39</td>
<td></td>
</tr>
<tr>
<td>Male/female (n/n)</td>
<td>8/4</td>
<td>4/6</td>
</tr>
<tr>
<td>Warfarin (mg/per week) mean±SD</td>
<td>34.16±10.53</td>
<td></td>
</tr>
<tr>
<td>Aspirin, n%</td>
<td>58.3</td>
<td></td>
</tr>
<tr>
<td>Statin, n%</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>16.6</td>
<td></td>
</tr>
</tbody>
</table>
values were determined using automatic CT function available. Relative gene expression levels for the three replications were calculated using the $2^{-\Delta\Delta CT}$ method \(^{16}\). Melting curve was used to determine the specific amplification of the specific gene segments.

**Data analysis**

The measurement of the cDNA samples was performed in duplicate. After obtaining the pair CT for each sample, their means were calculated. By importing the data to SPSS19, the outliers were first identified and excluded from the study along with their control samples. The results were measured as mean±SD. T-test and One-way ANOVA were used to compare the means between the two groups. Each test was repeated at least twice and performed independently.

**Results**

The fluorescent light was continuously monitored and the temperature was gradually increased from 60 to 95°C by the device. The melting curve of miR-133a-3p and snRNA-U6 was obtained as a single peak curve indicating the proliferation of the intended specific product and lack of its genomic DNA contamination (Figures 1A and 1B). Furthermore, the qPCR product was placed on 2% Agarose Gel. A molecular 50 bp DNA Ladder was used in the reaction above and fragments shorter than 50 base pairs were considered as specific targets. There was only one specific band (approximately 48 bp) in each of the reactions done with primers miR-133a-3p and snRNA-U6 confirming the specificity of PCR products in our sample (Figure 2).

A comparison of the mean of miR-133a-3p expression in patients receiving warfarin and the patients in control group demonstrated that the expression levels were higher in warfarin receivers indicating a significant difference between the two groups (p<0.01) (Figure 3).

A comparison of mean of miR-133a-3p expression rate between two different age groups receiving warfarin demonstrated that the expression levels were higher in age group (51-70) indicating a significant difference between the two groups (p<0.05) (Figure 4).
Other variables like warfarin dose, gender, INR and BMI were not significantly effective on the miR-133a-3p expression rate in individuals receiving warfarin.

Discussion
Due to individual differences, the patients’ response to warfarin is different. Some studies have indicated that at least 30% of the difference in dosage is attributed to genetic variants in CYP2C9 and VKORC1 genes. Also, 1-7% of dose changes required for warfarin is related to CYP4F2 gene. On the whole, 60% of the changes is attributable to a combination of various factors such as height, weight, and the genetic variants including VKORC1, CYP4F2, and CYP2C9. In any case, about 30-40% of drug dosage changes are not known yet.

It has been observed that diseases and different compounds affect the expression of miRNAs. Expression of multiple miRNAs in the circulation of the plaque for Unstable Angina (UA) patients showed the important roles of this drug on regulating signal pathways for the pathogenesis of UA. The expression levels of miR-22 and miR-133a-3p significantly increased and decreased, respectively by induction of cardiomyocyte hypertrophy by using angiotensin II (hypertrophy inducer). Nonetheless, after treatment of cardiomyocytes with atorvastatin, the expression level of miR-22 decreased significantly, but the expression level of miR-133a-3p did not change significantly which is in agreement with our results. The physical features of individuals (age, gender, and race) affect the expression of both mRNA and miRNA. It has been observed that the mRNA and miRNA content of platelet varies with respect to gender. Simon et al showed in their study that miR-223 is one of the nine miRNAs that are different in men and women. Interestingly, of seven mRNAs which vary in the two genders, three were the potential targets of miR-223.

In this study, different samples derived from different age group were used. Nevertheless, there was no significant difference between the groups of men and women who received warfarin in our study which could be caused by the nature and function of drudge. In this work, any correlation between miR-133a-3p expression and BMI was observed which is in agreement with similar works. There was a significant difference in miRNA expression level in our study between the two different age groups receiving warfarin. Regarding the profile expression of miRNAs related to age in regulating the expression of genes involved in drug metabolism, the results of two studies indicated that the expression of miRs-128, -106a, -18a, -484, 548a-3p, and 425 decreased significantly in both platelet and PBMC. It seems that a wide range of physiologic pathways contribute to the age and life span of individuals and it is believed that the genetics of age is complex. Normalization of miRNA expression with internal control is of utmost importance. Some studies show that the simultaneous use of several internal controls for exactly measuring the changes in expression is more suitable. Unfortunately, snRNA-U6 did not manifest a stable expression in our study. Regarding the role of VKORC1 gene in the coagulation pathway and prevention of vascular calcification, it seems that warfarin has some effect on the expression of miR-133a.

Furthermore, some studies have indicated that warfarin exerts an anticarcinogenic and anti-inflammatory effect through inhibiting proteins of the signaling pathway (mitogen-activated protein kinase (MAPK/ERK) that regulate various activities of this pathway including cell proliferation, mitosis, cell motility, metabolism, and apoptosis. It has been observed that warfarin in low concentrations has anti-inflammatory effects due to inhibition of secretion of IL-6 and the signaling path Tyro3/Gas6 while it is pre-inflammatory in high concentrations. It seems that miRNAs contribute to the regulation of genes involved in ADME of drugs under physiologic conditions like inflammation. Additionally, the regulation of the expression of other genes involved in warfarin metabolism like CYP2C9 gene by miR-130b related to inflammation has been indicated. Considering the controlling role of miR-133a-3p in the coagulation pathway, the recent result indicates that warfarin affects the expression of miR-133a. This expression may be potentially important for treatment by anticoagulants.

Conclusion
Awareness of miRNA expression patterns is important in identifying preventing and therapeutic strategies in patients receiving warfarin after mechanical heart valve replacement that are at risk of thrombosis and in selection of suitable dose of warfarin as a prognosis and diagnosis marker. An awareness of the time course of miRNA expression profile can improve efficiency of response to warfarin. Type of response to drug can help to effective treatment by an awareness of the course of expression of miRNAs. These results show that miRNAs as molecular interference regulators are involved in regulation and modulation of several genes implicated in homeostasis and hemostasis in normal, pathogenic and pharmaceutical conditions, so they influence gene expression of target genes.

Acknowledgement
We would like to thank Dr. Sabbagh for comments and help in submitting our paper.

References
2. Li Y, Zhu J, Ding JQ. VKORC1 rs2359612 and rs-9923231 polymorphisms correlate with high risks of car-


