Analysis of differential expression of miR-223 in platelets of elderly CHD patients before and after the autologous stem cell transplantation

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Abstract. – OBJECTIVE: The aim of this study was to investigate the differential expression of miR-223 in the platelets of elderly patients with coronary heart disease (CHD) before and after autologous stem cell transplantation.

PATIENTS AND METHODS: In this study, 26 elderly CHD patients were enrolled for treatment from February 2014 to August 2015. Elbow venous blood was collected before and after autologous stem cell transplantation, respectively. Phosphorylation levels of vasodilator stimulated phosphoprotein (VASP) of platelets were assayed before and after the treatment through the flow cytometer. The VASP and Ago-2 protein expression were detected by using ELISA and Western blot, respectively.

RESULTS: The differential expression of miR-223, VASP and Ago-2 in CHD patients before and after treatment were detected using qRT-PCR. The platelet aggregation rate in the blood of patients was measured before and after the treatment by a platelet aggregation test. Compared to the levels before the treatment, the results of flow cytometry revealed that the phosphorylation levels of VASP in platelet of CHD patients who received the autologous stem cell transplantation was significantly increased (p<0.05). Also, ELISA and Western blot results showed that the protein expression of Ago-2 in elderly patients that received the treatment was significantly increased at (2.36±0.17) µg/L. However, there was no statistically significant difference in the comparison of VASP protein expression before and after treatment (p>0.05). The results of qRT-PCR showed that the expressions of Ago-2 and miR-223 in elderly CHD patients were significantly increased after autologous stem cell transplantation compared to those before the treatment with statistically significant differences (p<0.05). However, there were no statistically significant differences identified in the comparison of the mRNA expression of the VASP gene before and after the treatment (p>0.05).

CONCLUSIONS: Therefore, the miR-223 expression of platelets was significantly decreased after elderly CHD patients received autologous stem cell transplantation. Moreover, it leads to a decrease in protein expression and phosphorylation levels of VASP in order to reduce the occurrence of platelet aggregation.

Key Words: CHD in the elderly, Autologous stem cell transplantation, Platelet aggregation, miR-223, Differential expression analysis.

Introduction

Epidemiology\(^1\) revealed that the onset of stroke and coronary heart disease (CHD) and the occurrence rates till 2015 in China, have become one of the major causes of death in middle-aged and elderly patients. In recent years, a gradual decrease has been seen in the occurrence rates of CHD and stroke in developed countries. However, CHD and stroke are still the major factors that threaten the health and lives of the elderly population. Statistical data\(^2\) indicated that there were about 600 million people suffering from CHD and stroke until 2015, which accounted for 32.15% of the elderly population. Also, the statistics of a number of CHD patients and stroke\(^3\) have shown that the occurrence rates of these two diseases in China are still increasing. CHD has become one of the chronic diseases that threaten the health of the elderly due to various factors, such as the long onset time and difficulties during treatment\(^4\). According to the clinical and relevant medical studies, the pathogenesis of CHD suggests that the atherosclerosis of coronary artery narrows or even blocks the lumen of the coronary artery, which leads to acute ischemia or hypoxia of myocardial cells\(^5\). As studies on CHD continued, it has been found that the platelets played an important role in the
acute thrombosis of CHD. For instance, the endothelium of vessels under an injured state can release the related proteins with adhesive activity. For the lack of nuclei and genomic DNA in platelets, it has been considered that there was no relevant regulation mechanism in platelets, and only mRNA generated from genetic expression at an early stage can be found inside cells. In recent years, research has identified that the main function of many miRNAs is to regulate gene expression. For example, studies have found that miRNAs played an important role in various processes, such as differentiation of embryonic stem cells and generation of erythrocytes. Also, studies have indicated that miR-223, as one of the miRNAs that are relatively high in platelets, can promote the maturation of miR-233 through the effect of the Ago-2 protein in platelets. The major mechanism of stem cell transplantation technique is to re-introduce healthy blood cells from the bone marrow with high self-regeneration and differentiation ability into the body, therefore to improve the hematopoietic dysfunction and abnormality in platelet aggregation in CHD patients. In this study, we performed miR-223 differential expression analysis before and after autologous stem cell transplantation treatment for elderly CHD patients to discover the correlation between miR-223 and platelet aggregation.

**Patients and Methods**

**Patients**

We selected 26 elderly CHD patients that were admitted to the hospital (Yishui Central Hospital, Linyi, Shandong, China) for treatment from February 2014 to August 2015. This study was approved by the Ethical Committee of Yishui Central Hospital, and all patients involved voluntarily signed the informed consent. There were 16 males and 10 females with an average age of (68.4±4.7) years old. 5 mL of elbow venous blood was collected before and after autologous stem cell transplantation and centrifuged at 1000 g at 4°C for 10 min with the supernatant. The blood cells and platelet were preserved in the liquid nitrogen for later experiments.

**qRT-PCR**

**RNA Extraction**

Approximately 0.2 g of the experimental sample was taken out from the -80°C freezer and placed in the prepared icebox for melting. 0.45 mL RNA plus was added to the sample after the most of it was melted. Then the experimental sample was blown using a pipette; samples were centrifuged at 12000 g at 4°C for 15 min and the supernatant was abandoned. 200 µl chloroform were added into the sediment, which was later acutely blown by pipette or shaken for 15 s and placed on ice for 15 min. Samples were centrifuged at 12000 g at 4°C for 15 min. The supernatant was drawn gently using a pipette and it was transferred to an EP tube containing RNase followed by the addition of equal volume of isopropanol. The tube was inverted several times rapidly to mix well and then placed on ice for 10 min. Samples were centrifuged at 12000 g at 4°C for 15 min. The supernatant was discarded, and 750 µl of 75% ethanol was added along the tube wall and slightly mixed well with the sample, which was followed by centrifugation at 12000 g at 4°C for 15 min. The supernatant was discarded, and most of the residual ethanol was eliminated (or placed at the room temperature for 15 to 20 min). There was an appropriate amount of RNase-free water added and the weight of the RNA was measured. The rest of RNA was used for the reverse-transcription.

**Flow Cytometry**

VASP phosphorylation levels were detected using flow cytometer according to the following
procedures. Firstly, 10 µL of reagent A was added into plastic tubes (T1-T3), and reagent B was added into the plastic tubes (T2-T3). Finally, 10 µL of patient blood before and after treatment was added into plastic tubes and placed at 37°C for 8 min. Then, the fixation was as follows: the prepared reagent C was added in the above different plastic tubes, respectively, to fixate the cells, and incubation for 5 min was carried out after the samples were mixed well using a pipette. Then, the cells were permeabilized and immunolabeled. 10 µL of reagent D (phosphorylated antibody of VASP and fracturing agent of membrane) was added into the T1-T2 plastic tubes, and incubated for 10 min. Finally, FCM analysis occurred. The response index of platelets was indirectly assessed through evaluating the VASP phosphorylation levels of VASP using an average of fluorescence strength calibrated under the static state and activated state.

**Enzyme-Linked Immune Sorbent Assay**

The procedures were performed according to the instructions of the ELISA kit (TaKaRa, Otsu, Shiga, Japan). In this study, the standard protein sample of ELISA was diluted 1:100 using the Assay Buffer accordingly, and the standard curve was prepared. The samples for the test were diluted 1:50 using phosphate-buffered saline (PBS) (pH = 7.2) and 100 µL of sample for test and 50 µL detection solution were added into each well in sequence, followed by incubation for 2 h at room temperature. Then, the 3,3′,5,5′-tetramethylbenzidine (TMB) color development substrate was added, and OD₄₉₅ was measured. Subsequently, contents and concentration of VASP and Ago-2 in each sample for the test were detected using a standard curve.

**Western-Blotting**

In this study, we extracted total protein from samples using the extraction kit for total protein of animals. The specific procedures were carried out according to the instructions. 0.5 mg of protein was accurately weighed from different samples, and quantitatively assayed using Coomassie brilliant blue staining after extraction of total protein. 20 µL of the treated sample was taken for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by membrane transferring and blocking for 2 h. Thereafter, the primary antibody was diluted in 1:1000 for incubation at room temperature for 2 h. Then, the secondary antibody was added for incubation at the room temperature for 2 h. Later, the membrane was rinsed using eluent for a total of 5 times (10 min/time) and a color-substrate solution was added for color development.

**Detecting the Platelet Aggregation Rate**

In this study, we detected the platelet aggregation rate in different research samples using the CHRONO-LOG (Havertown, PA, USA) Whole-Blood Aggregometer in the following procedures: the reaction cups of platelet connected or not connected with magnetic bar were placed in the pre-warmed wells for pre heat (37°C); the blood of patient in different research samples was centrifuged at 1000 g for 5 min; the samples obtained were added into pre-warmed reaction cups to assay for platelet aggregation rate with the blood sample collected before the treatment.

**Statistical Analysis**

Data was analyzed using the SPSS14 software (SPSS Inc., Chicago, IL, USA). The c²-test was performed for the comparison of categorical statistical data where α=0.05 was set as the significant level, and differences with p<0.05 were statistically significant; α=0.05 was set as the very significant level, and differences with p<0.01 were very statistically significant.

**Results**

**Differences of the Expressions of VASP, Ago-2 and miR-223 in elderly CHD Patients Before and After the Treatment**

With the blood samples collected from elderly CHD patients before and after the treatment, we extracted the total RNA from the cells. The differences that were lying among the mRNA expressions of different genes in different samples through qRT-PCR were also detected. The results as shown in Figure 1 suggested that compared
to the levels before treatment, the mRNA expressions of Ago-2 and miR-223 in patients received the autologous stem cell transplantation, were remarkably increased ($p<0.05$). The expression of Ago-2 was 37.4 times as that before treatment. The expression of miR-223 was 54.8 times higher than before the treatment, but there were no significant differences in the comparison of the mRNA expression of the VASP gene before and after treatment ($p>0.05$).

Differences of the Protein Expressions of VASP and Ago-2 in Elderly CHD Patients before and After the Treatment

We collected platelets from elderly CHD patients before and after the treatment. The total protein was extracted from inside the cells. The differences lying among the protein expressions of VASP and Ago-2 in different samples through ELISA were determined, as shown in Figure 2. There was no significant change in the comparison of protein expressions between the VASP gene before and after the treatment (1.65±0.11 µg/L vs. 1.59±0.13 µg/L). There were no statistically significant differences identified in comparison ($p>0.05$). However, the protein expression of Ago-2 after treatment (2.36±0.17 µg/L) was significantly higher than that before treatment (0.57±0.15 µg/L) with a statistically significant difference ($p<0.05$), which indicated that autologous stem cell transplantation could increase the protein expression of Ago-2.
miR-223 expression in platelet of CHD patients and stem cell transplantation

Figure 2. Differences of the protein expressions of VASP and Ago-2 in elderly CHD patients before and after the treatment.

to the protein expressions of VASP and Ago-2 before the treatment, (1.65±0.11, 0.57±0.15) µg/L, protein expression of VASP and Ago-2 after the autologous stem cell transplantation, (4.04±0.21, 2.36±0.17) µg/L, was remarkably elevated with statistically significant differences (p<0.05).

The changes of miR-233 in elderly CHD Patients over Time

To measure the influence of autologous stem cell transplantation for elderly CHD patients on miR-223 expression in platelets, the variations were detected in miR-223 expression in patients before and 36 months after the autologous stem cell transplantation through qRT-PCR. As shown in Figure 4, the miR-233 expression was determined in patients after stem cell transplantation was significantly augmented compared to that before treatment. Moreover, with the extension of time, an elevated but subsequently stabilized trend was identified in the expression.

Figure 3. Differences of the protein expressions of VASP and Ago-2 in elderly CHD patients before and after the treatment using Western blot. A, Color development result after electrophoresis on the gel in Western blot; B, Relative quantitative assay result through color development after electrophoresis on the gel in Western blot.

Detecting the Platelet Aggregation Rate of Elderly CHD Patients Before and After Treatment

The formation and exacerbation of thrombus, as a major factor leading to the death of elderly CHD patients, played an important role in this process. In this study, we detected the platelet aggregation rates before and after autologous stem cell transplantation using a platelet aggregation assay kit. We discovered that platelet aggregation

Figure 4. The changes of miR-233 in elderly CHD patients over time.
in elderly CHD patients after autologous stem cell transplantation was significantly decreased compared to that before treatment as shown in Figure 5. The results indicated that the autologous stem cell transplantation can significantly decrease the occurrence of platelet aggregation in elderly CHD patients and reduce the risk of thrombosis in patients.

Detecting the Phosphorylation Level of VASP in Elderly CHD Patients Before and After Treatment

The phosphorylated vasodilator stimulated phosphoprotein (VASP) can promote vasodilation, which further decreased the occurrence of platelet aggregation. The phosphorylation state of VASP using flow cytometer to reflect platelet reactivity index (PRI) was detected. As shown in Figure 6, the phosphorylation levels of VASP in elderly CHD patients after the autologous stem cell transplantation (45.32%), were significantly elevated compared to that before treatment with a statistically significant difference \((p<0.05)\).

Discussion

In recent years, it has been discovered that miRNA (microRNA) was a type of single-chain RNA fragment in human cells with a length of 70-90 bases, which cannot be translated into amino acids\(^\text{13}\). Currently, miRNAs have been gradually proven to play an important role in regulating the genetic expression. For example, Zhao et al\(^\text{14}\) have found that miRNAs \(\text{in vivo}\), under the effect of RNA polymerase, was firstly transcribed into an immature RNA fragment with a cap sequence in the 5’ end and a polynucleotide sequence in the 3’ end. Under the effect of RNaseIII Drosha enzyme and other relevant cofactors, it was spliced into the precursor of miRNA (70 to 90 nt) with a hairpin structure\(^\text{15}\). Subsequently, the precursor will be transported into the cytoplasm and processed by other relevant enzymes into the mature miRNA with regulatory functions\(^\text{16}\). Some scholars\(^\text{17}\) have found that miRNAs can participate in the regulation and expression of multiple genes. It has been found that miRNAs can take part in the regulation of relevant oncogenes of various cancers, such as breast and colorectal cancer\(^\text{18}\). Studies of miRNAs\(^\text{19,20}\) have concluded that multiple types of pri-miRNAs, which existed in platelets, can be catalyzed by various proteins in platelet, such as Ago-2, into the miRNA with certain activity. Li et al\(^\text{21}\) have suggested that Ago-2 protein, as a major protein to accelerate miRNAs, exerts its effect. It can bind with miRNA-223 to promote the maturation of miR-223. However, through the analysis of proteins in platelets, it has been discovered that

\[\text{Figure 5. Detecting the platelet aggregation rate of elderly CHD patients before and after treatment.}\]

\[\text{Figure 6. Phosphorylation level of VASP in elderly CHD patients before and after treatment.}\]
miR-223 expression in platelet of CHD patients and stem cell transplantation

platelets only had the immature mRNA, instead of the complete genome. Therefore, the mature miRNA-223 may regulate gene expression in the platelets through the interaction with immature miRNA. In this work, we found that there was a gradual increasing trend in elderly patients who received the autologous stem cell transplantation and there was no statistically significant change found in protein expression in the detection of VASP protein content. However, a significantly increasing trend was shown in the content of phosphorylated protein after autologous stem cell transplantation. Simultaneously, the protein expression of Ago-2, as an important protein that accelerated the maturation of miRNA, was significantly elevated after treatment. It indicated that the stem cell transplantation can simultaneously augment the phosphorylation levels of VASP in platelet and promote the expression of Ago-2. In the assay of platelet aggregation rates in elderly CHD patients before and after treatment, we discovered that the platelet aggregation rate was significantly decreased after stem cell transplantation, while the platelet aggregation was the major promoting factor in the occurrence of CHD in elderly patients. The decrease of platelet aggregation rate could reflect the alleviation of the disease.

Conclusions

For the first time, we discovered that stem cell transplantation can reduce the occurrence of platelet aggregation through an increase in the content of miRNA-223 in the patient. However, the main factors leading to an increase of miRNA-223 expression were elevations in the protein expression of Ago-2 and the levels of phosphorylated VASP.

Conflict of interest

The authors declare no conflicts of interest.

References


