

Original Article

Role of peripheral blood microRNA-181b in acute vascular rejection after renal transplantation

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Received November 3, 2017; Accepted December 8, 2017; Epub February 15, 2018; Published February 28, 2018

Abstract: Objective: To investigate the role of peripheral blood microRNA-181b in acute vascular rejection after renal transplantation. Methods: Thirty-four patients admitted to our hospital for initial renal transplantation from January 2013 to December 2016 were enrolled in this study. The patients were assigned to either the acute vascular rejection group (n=14) or the non-acute vascular rejection group (n=20) according to the acute vascular rejection in their transplanted kidneys. The miR-181b levels in peripheral blood of all the patients were detected using real-time polymerase chain reaction (PCR) preoperatively, at 1 week, 2, 3, and 4 weeks postoperatively, respectively. Normal peripheral blood mononuclear cells were stratified into the active subgroup and the inactive subgroup according to phytohemagglutinin stimulation profiles. Expression levels of miR-181b were detected by the real-time PCR and compared between the two subgroups. Patients with acute vascular rejection were stratified into the steroid-sensitive subgroup and the steroid-tolerable subgroup according to their sensitivity to hormone therapy after 2 weeks of hormone pulse therapy. And the real-time PCR was applied to detect and compare the expression levels of peripheral blood miR-181b of both groups. The receiver operating characteristic (ROC) curve was used to analyze and evaluate the predictive value of peripheral blood miR-181b in acute vascular rejection after renal transplantation. Results: Expression levels of peripheral blood miR-181b in the acute vascular rejection group were significantly lower than those of the non-acute vascular rejection group at different time points of 1, 2, 3, and 4 weeks, postoperatively (all $P < 0.001$). The expression level of miR-181b in peripheral blood mononuclear cells activated by phytohemagglutinin was significantly lower than that in the inactive subgroup (1.05 ± 0.11 vs 0.36 ± 0.09 , $P < 0.001$). As compared with the steroid-tolerable subgroup, the expression level of miR-181b in the steroid-sensitive subgroup was markedly higher (0.48 ± 0.07 vs 0.64 ± 0.08 , $P < 0.001$). The ROC curve showed that the detection of expression level of miR-181b was a predictor of acute vascular rejection after renal transplantation. Conclusion: The expression level of peripheral blood miR-181b was reduced in the transplanted kidney with acute vascular rejection. Monitoring of the miR-181b levels could be one of the markers for assessment of acute vascular rejection after renal transplantation.

Keywords: Renal transplantation, acute vascular rejection, miR-181b

Introduction

MicroRNA is a small, endogenous non-coding single stranded RNA molecule, which is evolved conservatively. Its mechanisms of action are to completely or incompletely pair with 3'UTR end of target mRNA, and suppress target mRNA translation or degradation, which in turn regulates proliferation, migration, differentiation, growth and development and other vital activities of cells [1, 2]. According to some studies, abnormal expression of microRNA is closely

associated with the onset and progression of multiple diseases [3-5]. Many studies have demonstrated that miRNA plays a vital role in acute rejection after renal transplantation [6]. Although the proportion of patients with vascular rejection is not high, it is difficult to confirm acute vascular rejection, and the efficacy of its treatment is poor. If vascular rejection is not diagnosed at early stage, it would cause renal injury, which will lead to renal dysfunction and a reduced survival rate of renal transplantation. Of note, accurate diagnosis of vascular rejection

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Table 1. Comparison of clinical information between two groups before transplantation

Variable	CN	M/F (n)	AA (year)	HLA MN	MDC (year)	BUN (mmol/L)	Cr (μ mol/L)	CCr (mL/min)	UPr (g/d)
AVRRG	14	6/8	37.5 \pm 4.1	2.4 \pm 0.6	2.1 \pm 0.9	23.9 \pm 7.8	372.5 \pm 86.1	31.1 \pm 8.5	1.2 \pm 0.7
NVRRG	20	10/10	36.1 \pm 3.8	2.2 \pm 0.5	1.9 \pm 0.7	22.7 \pm 7.2	364.5 \pm 79.3	32.4 \pm 8.9	1.3 \pm 0.8
t/ χ^2		1.865	1.547	1.603	2.212	1.714	1.921	2.408	2.611
P		0.613	0.677	0.659	0.454	0.642	0.514	0.394	0.298

Note: AVRRG denotes acute vascular rejection group, NVRRG non-acute vascular rejection group, CN case number, M male, F female, AA average age, HLA MN HLA mismatch number, MDC mean disease course, BUN blood urea nitrogen, Cr serum creatinine, CCr creatinine clearance rate, UPr urinary protein quantification.

tion after renal transplantation is of significance before the onset of irreversible injury in the kidney.

As a relatively noninvasive examination, the miRNA levels in the peripheral blood have been drawing increasing attention from clinicians. MiR-181b is expressed in all tissues of human body and its expression level is high in the heart, the brain, the lung and the thymus gland [7]. Some studies have showed that miR-181b reduces inflammatory response by regulating expression of target genes in vascular endothelial cells [8, 9]. Additionally, miR-181b plays a crucial role in immune tolerance, development of T or B cells and differentiation of NK (natural killer) cells [10-13]. However, the roles and mechanisms of miR-181b in the onset and progression of acute vascular rejection after renal transplantation remain unclear. Therefore, the aim of this study was to examine the changes in the miR-181b levels in the peripheral blood of patients with acute vascular rejection after renal transplantation, and to analyze the correlation between changes in the miR-181b levels and acute vascular rejection.

Materials and methods

General information

Thirty-four patients undergoing the initial renal transplantation in our hospital from January 2013 to December 2016 were recruited in this study. Among them, 16 were male and 18 were female, with an age ranging from 20 to 55 years (mean, 36.8 \pm 4.6 years). The blood types of donors and receivers were matched, they were lymphocytotoxicity antibody-native and panel reactive antibody-negative. For maintenance of immunosuppression, a triple regimen consisting of cyclosporine/tacrolimus, mycophenolate mofetil and prednisone was used after renal

transplantation. According to whether acute vascular rejection occurred within 6 months after renal transplantation or not, the patients were assigned to the acute vascular rejection group (n=14) or the non-acute rejection group (n=20). Diagnosis of acute vascular rejection should meet the following conditions: Fever, elevated blood pressure, decreased urine volume, swelling pain in the transplanted kidney area, increased urine protein, elevated serum creatinine levels and other clinical manifestations; increased arterial vascular resistance indexes on all scales of transplanted kidney, reduced blood flow and enlarged transplanted kidney as demonstrated by color Doppler ultrasonography. Patients who were suspected to have acute vascular rejection were recommended for puncture biopsy of the kidney, and their pathological biopsy suggested fundamental pathological changes of acute vascular rejection [14]. Once the patients were diagnosed as having acute vascular rejection after renal transplantation, methylprednisolone pulse therapy was immediately applied, with 3 days of consecutive injection at the doses of 0.5 g, 0.25 g and 0.25 g respectively. After two weeks of hormone pulse therapy, the patients with acute vascular rejection whose above conditions didn't improve significantly were diagnosed as having hormone resistance. The patients with acute vascular rejection were stratified into two subgroups: the steroid-tolerable subgroup and the steroid-sensitive subgroup; the real-time polymerase chain reaction (PCR) was utilized to detect and analyze expression levels of miR-181b in the peripheral blood of both subgroups. Inclusion criteria: Patients who were older than 18 years old, had renal transplantation for the first time and were followed up for 6 months. Exclusion criteria: Patients who had other basic disease, infection to surgical incision, pulmonary infection or

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other infectious disease, renal dysfunction caused by drug toxicity and urinary tract obstruction or renal arterial stenosis. The study was approved by the Hospital Ethics Committee, and obtained written informed consents from all the patients.

Real-time quantitative PCR detection for miR-181b level in the peripheral blood

Peripheral blood (5 mL) of patients from different groups was extracted at different time points (preoperatively, at 1 week, 2, 3, and 4 weeks postoperatively). And miRNA extraction was performed following the instructions on the mirVana miRNA isolation kit. The extracted miRNAs were reversely transcribed into cDNA by the TaqMan MicroRNA reverse transcription kit. Then miR-181b or internal reference U6 primer was added to achieve amplification. The reaction conditions were pre-denaturation at 95°C for 10 min, denaturation at 95°C for 15 s, renaturation at 60°C for 1 min and a final extension at 72°C for 1 min, with a total of 40 cycles. After PCR was completed, corresponding Ct values were obtained by analyzing gene amplification on the ABI 7300 system software. U6 was used as internal reference to adjust the copy numbers of PCR template. And relative gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method.

Culture and activation of peripheral blood mononuclear cells

The peripheral blood samples (5 mL for each) were drawn from ten patients who were healthy as demonstrated by physical examination. The standard Ficoll gradient centrifugation and subsequent enrichment were used for extraction of peripheral blood mononuclear cells, which were then resuspended in PRMI1640 culture solution that contained 10% of fetal bovine serum, with the readjusted concentration of 1×10^6 /mL. After that, the cells were inoculated in culture flasks. The mononuclear cells from the 10 patients were randomly stratified into the active subgroup (n=5) and the inactive subgroup (n=5). Phytohemagglutinin (200 mg/L) was added into the active subgroup to stimulate activation of mononuclear cells, and the same dose of culture solution was added into the inactive subgroups as controls. Then, the cells were cultured in incubators at 37°C with 5% CO₂ for 72 h. And the real-time quantitative PCR detection was used to examine miR-181b

levels of mononuclear cells in the two subgroups.

Statistical analyses

SPSS software (version 21.0) was used for statistical analysis of all the data. Measurement data were represented as mean \pm standard deviation, with the t-test for inter-group comparison. Enumeration data were expressed as percentage, with the chi-square test for inter-group comparisons. The receiver operating characteristics (ROC) curve was established, and the role of the peripheral blood miR-181b in prediction of acute vascular rejection after renal transplantation was assessed according to the area under the curve. $P < 0.05$ was deemed to be statistically different.

Results

Comparison of general information between two groups

In the acute vascular rejection group, 6 patients were male and 8 were female, with a mean age of 37.5 ± 4.1 years, HLA mismatch number of 2.4 ± 0.6 , the mean course of disease of 2.1 ± 0.9 years, the blood urea nitrogen before transplantation of 23.9 ± 7.8 mmol/L, the serum creatinine levels of 372.5 ± 86.1 μ mol/L, the creatinine clearance rate of 31.1 ± 8.5 mL/min, and the urinary protein quantification at 1.2 ± 0.7 g/d. In the non-acute vascular rejection group, 10 patients were male and 10 were female, with an average age of 36.1 ± 3.8 years, HLA mismatch number of 2.2 ± 0.5 , the mean course of disease of 1.9 ± 0.7 years, the blood urea nitrogen before transplantation of 22.7 ± 7.2 mmol/L, the serum creatinine levels of 364.5 ± 79.3 μ mol/L, the creatinine clearance rate of 32.4 ± 8.9 mL/min, and the urinary protein quantification of 1.3 ± 0.8 g/d. The differences in sex, age, HLA mismatch number, time of disease course and renal function before transplantation were statistically insignificant ($P > 0.05$), as shown in **Table 1**.

Comparison of expression levels of peripheral blood miR-181b between two groups

The preoperative miR-181b levels in the peripheral blood were insignificantly different between the two groups ($P > 0.05$; 1.07 ± 0.09 vs 1.09 ± 0.08 , $t = 0.512$, $P = 0.639$). At different time points of postoperative 1, 2, 3, and 4 weeks, the expression levels of peripheral blood miR-

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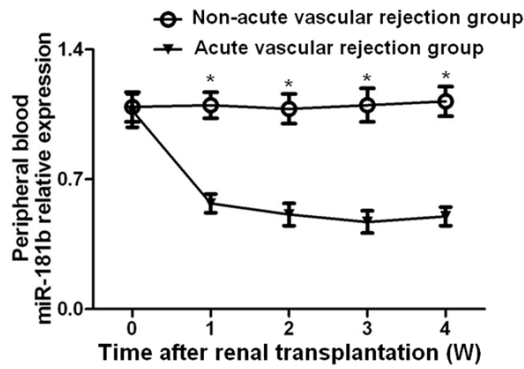


Figure 1. Comparison of expression levels of peripheral blood miR-181b at different time points before and after transplantation between the two groups. Compared with acute vascular rejection group, * $P < 0.001$.

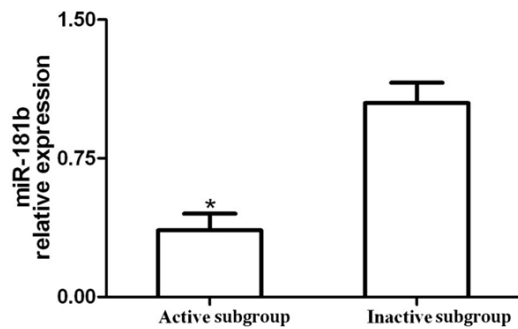


Figure 2. Comparison of expression levels of miR-181b between the active subgroup and the inactive subgroup. Compared with the inactive subgroup, * $P < 0.001$.

miR-181b in the acute vascular rejection group were markedly decreased, as 0.52 times (0.57 ± 0.05 vs 1.10 ± 0.07 , $t=21.342$, $P < 0.001$), 0.47 times (0.51 ± 0.06 vs 1.08 ± 0.08 , $t=22.528$, $P < 0.001$), 0.42 times (0.47 ± 0.06 vs 1.10 ± 0.09 , $t=20.779$, $P < 0.001$) and 0.45 times (0.50 ± 0.05 vs 1.12 ± 0.08 , $t=24.189$, $P < 0.001$) low respectively as those in the non-acute vascular rejection group. The differences in expression levels of peripheral blood miR-181b were statistically significant between the two groups at different time points after transplantation ($P < 0.001$, **Figure 1**).

Detection of expression levels of miR-181b in peripheral blood mononuclear cells

The cultured peripheral blood mononuclear cells of healthy subjects were stimulated with phytohemagglutinin and further cultured for 72 h. Compared with the inactive subgroup, the

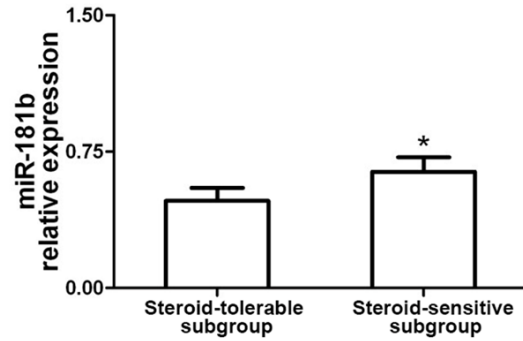


Figure 3. Comparison of the expression levels of miR-181b between the steroid-tolerable subgroup and the steroid-sensitive subgroup. Compared with the steroid-tolerable subgroup, * $P < 0.001$.

expression level of miR-181b in the active subgroup was significantly decreased (1.05 ± 0.11 vs 0.36 ± 0.09 , $P < 0.001$), as seen in **Figure 2**.

Comparison of expression levels of miR-181b between the steroid-tolerable subgroup and the steroid-sensitive subgroup

Among 14 patients who had acute vascular rejection after renal transplantation, 9 patients were sensitive to hormone therapy and 5 were insensitive. After two weeks of hormone pulse therapy, the expression level of miR-181b in the steroid-sensitive subgroup was significantly elevated when compared with that of the steroid-tolerable subgroup (0.48 ± 0.07 vs 0.64 ± 0.08 ; $P < 0.001$; **Figure 3**).

Results of the ROC curve for prediction of acute vascular rejection after renal transplantation by the miR-181b levels

The results of the ROC curve for prediction of acute vascular rejection after renal transplantation by the miR-181b levels showed that the area under the curve was 0.708, with statistical difference ($P = 0.002$). It suggests that the expression levels of miR-181b is a predictor for acute vascular rejection after renal transplantation, as shown in **Table 2** and **Figure 4**.

Discussion

Renal transplantation is the most effective treatment for patients with end-stage renal disease, but acute rejection is one of the key factors affecting renal transplantation. In acute vascular rejection, the vessels in the transplanted kidney are primarily injured tissues

Table 2. Results of the ROC curve for prediction of acute vascular rejection after renal transplantation by the miR-181b levels

Indicator	Area under the curve	Standard error	P	95% CI
MiR-181b	0.708	0.054	0.002	0.583-0.926

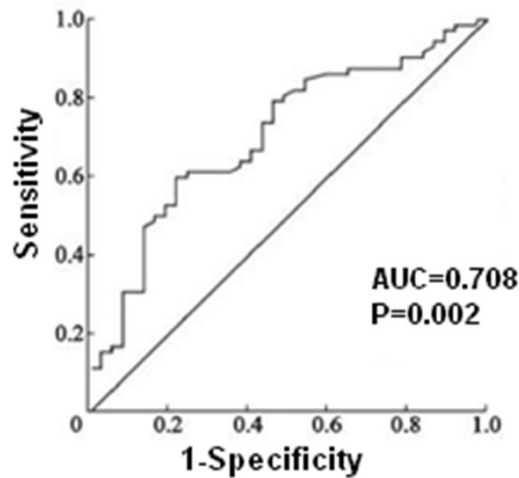


Figure 4. The ROC curve for prediction of acute vascular rejection after renal transplantation by the expression levels of miR-181b.

which have the pathological changes including vascular embolization, inflammation, swelling and necrosis of vascular endothelial cells, intravascular fibrin deposition and platelet aggregation, and infiltration of intravascular lymphocytes and neutrophils. Early diagnosis and timely treatment of acute vascular rejection effectively reduce the degrees of damages to the transplanted kidney and prolong its survival.

Peripheral blood miRNA is an ideal marker for its easy access, stable physical and chemical properties, good timeliness, and effective dynamic monitoring of occurrence, development and efficacy of diseases [15-17]. Multiple studies have showed that miRNA plays a regulatory role in acute rejection of renal transplantation, and miRNA detection effectively predicts acute rejection after renal transplantation [18, 19]. According to a study, the levels of miR-155, miR-142-5p and miR-122 were high in renal tissues and peripheral blood mononuclear cells in the transplanted kidney with acute vascular rejection [20]. MiR-10b promoted release of inflammatory cytokines and apoptosis of glomerular endothelial cells by suppress-

ing the BCL-2-L11 expressions, thereby involving in the occurrence of acute rejection in the transplanted kidney [21]. It could be seen that acute rejection in the trans-

planted kidney is closely related to abnormal expression of miRNA. And miRNA can be used as a biomarker to predict diagnosis and prognosis of acute rejection in the transplanted kidney.

MiR-181b is a member of the miR-181 family. Currently, studies are relatively rare on the regulatory role of miR-181b in the occurrence and development of disease. Some studies have showed that miR-181b regulates differentiation of vascular endothelial cells of primitive embryo [22]. Overexpression of miR-181b could reduce NF- κ B signaling activation and expression of target genes in vascular endothelial cells [23]. Animal experimental studies showed that inflammatory stimulation could reduce expression of miR-181b in mice. Injection of miR-181b in mice with endotoxemia could reduce activation of downstream signals of endothelial NF- κ B, inflow of cells and pulmonary injury [8]. Of note, miR-181b might be related to the occurrence of inflammatory diseases associated with the injury to vascular endothelial cells. In addition, miR-181 has an effect on the development of T cells. Studies presented that knockout of miR-181 could damage signaling pathway of PI3K and cause severe restriction to homeostasis of T cells [24]. The above studies indicate that miR-181b plays roles in vascular inflammation and the immune system.

The reports on the role of miR-181b in immune rejection after organ transplantation are rare and the role of miR-181b in acute vascular rejection after renal transplantation has never been reported. The results of our current study showed that when compared with the patients with non-acute vascular rejection after renal transplantation, those with acute vascular rejection after renal transplantation had significantly lower expression levels of miR-181b at different time points. Additionally, the expression level of peripheral blood miR-181b was maintained at a low level in patients with acute vascular reject reaction after operation, which indicates that the decrease in expression of peripheral blood miR-181b is significantly asso-

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ciated with the occurrence of acute vascular rejection after renal transplantation. In our current study, we also detected the expression levels of miR-181b in the steroid-sensitive subgroup and the steroid-tolerable subgroup after hormone pulse therapy. The results showed that the expression level of peripheral blood miR-181b of patients in the steroid-sensitive subgroup was significantly higher than that of the patients in the steroid-tolerable subgroup. It suggests that acute vascular rejection of patients improved after hormone pulse therapy, so the expression levels of peripheral blood miR-181b were elevated. In addition, we also examined the expression levels of miR-181b of peripheral mononuclear cells in active and inactive states. The results showed that the expression level of miR-181b in the active subgroup was significantly lower than that in the inactive subgroup. It indicates that miR-181b might be related to activation of immune cells. MiR-181b might further mediate the occurrence of postoperative acute vascular rejection by regulating growth, development, and inflammation of various immune cells. The above results demonstrate that miR-181b actually plays a key role in acute vascular rejection after renal transplantation, and the decrease in the expression levels of peripheral blood miR-181b is a marker to predict acute vascular rejection after renal transplantation.

In summary, our current study confirmed that the levels of miR-181b were reduced in acute vascular rejection after renal transplantation. Detection of miR-181b was helpful for improving diagnosis of acute vascular rejection after renal transplantation, and it had clinical value for guidance of treatment and evaluation of prognosis. The role of miR-181b in long-term prognosis after renal transplantation needs to be further studied due to the small sample size, short follow-up and other limits of our current study. The possible mechanisms of miR-181b in acute vascular rejection after renal transplantation are also one of the directions in the future relevant studies.

Declaration of conflict of interest

None.

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