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Original Article

Correlation between miRNA-126 expression abnormality and hypo-methylation of DNA in T cells of rheumatoid arthritis patients with respiratory failure

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Abstract: Rheumatoid arthritis is frequently complicated with respiratory failure, thus threatening patients' lives. Hypo-methylation of T cells plays a critical role in multiple diseases. DNMT1 is important for gene methylation and is the target gene of microRNA (miR)-126, which thus might regulate T cell gene methylation. This study, therefore, examined the expression of miR-126 during DNG hypo-methylation in T cells from rheumatoid arthritis patients, to illustrate the relationship between two factors. 33 rheumatoid arthritis patients complicated with respiratory failure were recruited based on ACR criteria, in parallel with 33 healthy individuals. 5 ml venous blood samples were collected to sort out T cells, whose RNA was extracted to quantify miR-126 and DNMT1 expression by qRT-PCR. Methylation test kit was used to reveal the change of methylation level of EGFL7 in T cells. T cell sorting had higher than 98% of purity in this study. Expression of miR-126 in study group was about 3.32 folds of control group, with significant difference ($P<0.05$). DNMT1 expression was remarkably lower than control group ($P<0.05$), and was negatively correlated with miR-126 expression ($r=0.643$, $P=0.021$). Methylation level of EGFL7 in disease group was significantly decreased compared to control group. Disease group showed lower DNA methylation level in T cells. In rheumatoid arthritis patients, miR-126 expression is negatively correlated with T cell methylation, probably via inhibition on DNMT1 expression after miR-126 up-regulation, causing hypo-methylation of T cell DNA. Elevated EGFL7 methylation further caused hypo-methylation of T cells via up-regulating miR-126.

Keywords: MiR-126, rheumatoid arthritis, respiratory failure, t cells, methylation

Introduction

Rheumatoid arthritis (RA) severely affects patient's life quality. RA frequently occurs in joint tissues and peripheral sites, and is presented as non-necrotic inflammation, showing symmetrical and chronic disease course. RA frequently causes multi-organ damage in the body. Auto-antibody can be identified from patient's serum, classifying it into one auto-immune disease. RA commonly occurs in people aging between 20 and 40 years, with more female patients. Complicated factors underlie RA pathogenesis, including endocrine, infectious, genetics, immune and other factors. Previous studies reported the critical role of abnormal auto-immunity in RA pathogenesis [1-4].

DNA methylation is one common scenario as it can directly change gene expressional profiles, and is correlated with various auto-immune diseases, such as systemic lupus erythematosus (SLE), psoriasis and RA [5]. Certain type of methylation is one active gene regulation mechanism, whilst others are the result of passive regulation. The major course of methylation is the selective addition of methyl group in cytosine of DNA molecule under catalyze of methyltransferase, further changing the structure of chromatin and interrupting DNA structural stability, altering DNA conformation or its interaction with proteins, thus achieving the goal of gene expression regulation. It has been known that maintenance of DNA methyl-transferase activity is critical during methylation process, as it can participate in the methylation of newly-synthesized strand in double stranded DNA, or completely methylate the whole DNA molecule on the un-methylated strand [6-8].

MicroRNA (miR) can regulate gene expression and exert critical regulatory roles in immune

disease. Previous study found that miR-126 in SLE patients played a role in T cell methylation via mediating DNMT1 expression. Bioinformatics analysis found that EGFL7 was the host gene of miR-126, between which certain correlation existed. A positive correlation also exists between miR-126 expression level and methylation level of EGFL7 [9-11]. Currently, few studies have been made regarding miR-126 expressional regulation and T cell methylation level in RA patients with respiratory failure. The study on this issue, therefore, can benefit the illustration of autoimmune diseases pathogenesis, and provide reliable targets for clinical treatment, as well as new insights for similar diseases.

Materials and methods

Reagent and materials

Mononuclear cell separation buffer (density gradient approach) was produced by Haoyang Bio (China). Total RNA extract kit was purchased from MOBIO (US). Pre-mixed Taq polymerase for PCR was produced by Tiangen Bio (China). Reagents for qRT-PCR were purchased from Promega (US). Hydrosulfite modification kit was purchased from Kangjing Bio (China). DNA recycling kit was produced by Kangwei (China). Other materials were purchased from Axygen (US). Nucleic acid analyzer was produced by Bio-Rad (US). Flow cytometry was purchased from BD (US).

General information of patients

A total of 33 RA patients who were admitted in the third affiliated hospital of Guangzhou medical university and were diagnosed with respiratory failure according to ACR criteria were recruited in the study group from August 2015 to July 2016. Average age of patients was 33.32±4.68 years, with disease course ranging from 3 to 8 years. 33 healthy individuals with normal blood test and without infectious disease were recruited in the control group. Average age of control group was 32.32±4.64 years. 5 ml fasted blood samples were collected from both groups and were stored in anticoagulation tube under 4°C for further use.

This study has been pre-approved by the ethical committee of the third affiliated hospital of Guangzhou medical university. All subjects have signed the consent forms before recruitment in this study.

T cell separation and assay

5 ml heparin-treated peripheral blood samples were inverted and mixed at room temperature. 25 ml gradient separation buffer was added into the centrifugation tube, with 30 ml blood samples covered on its surface. The centrifuge tube was carefully loaded for 1800 rpm centrifugation in 20 min. The second layer of grey-white phase was saved as mononuclear cells, which were transferred and re-suspended in 5 ml PBS. After centrifugation rinsing, cells were precipitated by 1800 rpm for 10 min, and were re-suspended in 0.1 M PBS containing 2% FCS. Magnetic beads (CD4+, CD25+) were then used for T cell sorting. In brief, 0.2 ml reagent A was slowly mixed with 1 ml buffer. The tube was put onto the magnetic scaffold for 30 s to remove the supernatant. The beads were then re-suspended in buffer liquid, and were mixed at 4°C for 30 min. Separation by magnetic field was applied for 2-3 min. PBS buffer was used to repeatedly rinse magnetic bead-cell complex, followed by the addition of 100 μl reagent B. Under room temperature incubation for 60 min, re-absorption reaction was performed for 2 min in a magnetic field. The supernatant was transferred to a new tube. Cells were collected after three times of rinsing. Flow cytometry was used to examine cell purity.

Cell RNA extraction and cDNA synthesis

Extraction of cellular RNA followed manual instruction of test kit. 100 μl cell suspension was firstly collected into RNAse-free tubes, which also contained 350 μl cell lysis buffer. To ensure complete lysis of those cells, the mixture was shaken for 15 s, with the addition of 200 μl absolute ethanol for inversion. The mixture was then applied into the middle of affinity column, which was centrifuged at 8000 rpm for 2-3 min. Liquids in the collection tube was discarded. The upper segment of column was added with 500 μl rinsing buffer, followed by 1 min centrifugation at the same speed. After two rounds of rinsing, the column was further centrifuged for 2 min without buffer in to remove ethanol in the rinsing buffer. The affinity column was opened and air-dried. 50 μl elution buffer was then applied, followed by RNA extraction under 14000 rpm for 2 min centrifu-

Table 1. Primer sequence

Name	Primer sequence (5'-3')
DNMT1 forward	CCCGCATCCCAGGACCTCTCT
DNMT1 reverse	CGGGGGACTGGCGA
mir-126 forward	CTAAGACCTGTGGAATGGC
mir-126 reverse	CTCAAAGATGTCATTGCC
β-actin forward	AACAGTCCGCCTAGAAGCAC
β-actin reverse	CGTTGACATCCGTAAAGA
EGFL7 forward	CTGCTGATGTGGCTTCTGGT
EGFL7 reverse	GAGGAAGGGCTGGTACACACPCR

gation. 1 μl RNA was used for further cDNA synthesis.

cDNA synthesis: 1 μg RNA and 1 μl primer were added into RNase-free tube, plus DEPC-treated water. After gentle mixture, incubation was performed at 70°C for 10 min. The mixture was placed on ice for 5-10 min incubation. 4 μl reverse transcriptase buffer, 2 μl DTT and 1 μl dNTPs were mixed for each reaction, and was added into RNA mixture. Reverse transcription was performed in PCR cycler at 42°C for 5 min. 1 μl reverse transcriptase was added for 42°C incubation for 1 h. Enzymatic reaction was quenched at 65°C. cDNA was stored at -80°C for further use.

qRT-PCR for miR-126 and DNMT1 expression

Using miR-126 and DNMT1 gene sequence as the template, Primer 5.0 software was used to design the primer. After homologous screening in NCBI BLAST, specific primer was used as shown in **Table 1**. Primers were synthesized by Sangon (China). Using β-actin as the internal reference, a 20 μl qRT-PCR system was prepared including 10 μl 2× Master Mix, 0.08 μM forward and reverse primers, 2 μl cDNA, 0.4 μl Taq polymerase and distilled water. The mixture was briefly centrifuged for 15 s for loading and test under the following conditions: 94°C pre-denature for 5 min, followed by 45 cycles each containing 95°C denature for 12 s, and 63°C annealing for 40 s. Each sample was tested in triplicates to obtain average expression values.

EGFL7 methylation test

EGFK7 methylation level was tested by sequencing after bisulfite modification. T cell genome was extracted and tested for nucleic acid concentration. Those samples with A260/A280 ratio between 1.8-1.9 were used to make sure no RNA or protein contamination. EGFL7 gene amplification was performed using routine PCR method using primers as shown in **Table 1**. Annealing conditions of PCR amplification were controlled at 60°C for 40 s in 30 cycles. PCR products were extracted by agarose gel electrophoresis. Bisulfite modification was used to modify extracted genomic DNA. In brief, 2 μg DNA was mixed with distilled water to 50 μl, plus 5.5 μl freshly prepared 3 M NaOH. After 42°C water bath for 30 min, 30 μl freshly prepared 10 mM hydroquinone was added into the mixture until the color turned into yellow. 3.6 M sodium bisulfide was then added to adjust pH to 5.0 and final volume to 5 ml. 200 μl paraffin oil was added to prevent evaporation and oxidation. 50°C water-bath was applied for 16 h. Samples were then tested for methylation by MeDIP-Seq.

Assay for cellular DNA methylation

T cell DNA methylation was tested using methylation specific AP-PCR in a total volume of 20 μl. Primer sequence was AACCC TCACC CTAAC CC. PCR conditions were: 95°C for 3 min, followed by 30 cycles each containing 95°C 30 s, 55°C 30 s, 68°C 90 s. PCR products were separated in 1.5% agarose gel electrophoresis. Methylation level of genome was deduced by the electrophoresis density, as higher fluorescent density indicated higher DNA methylation level.

Analysis and statistics of data

Experimental data were collected by Excel and analyzed in SPSS13.0 software. Analysis of variance was performed for statistical significance, when $P<0.05$ was reached.

Results

Assay for purity of T cell separation

Flow cytometry was used to test T cell purity. As shown in **Figure 1**, purity of extracted T cells was higher than 98%, suggesting that T cells acquired satisfied requirement of research.

qRT-PCR for miR-126 expression

Both disease and control group were tested under identical conditions. Each sample was

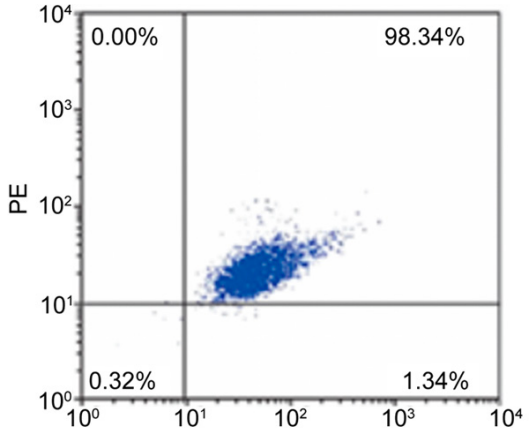

Figure 1. Flow cytometry for T cell purity.

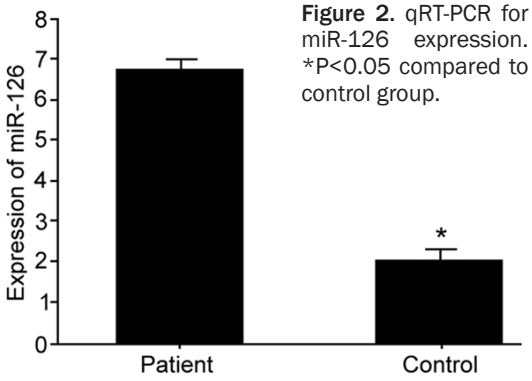

Figure 2. qRT-PCR for miR-126 expression. *P<0.05 compared to control group.

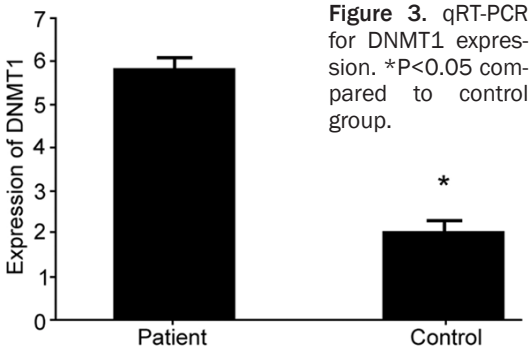

Figure 3. qRT-PCR for DNMT1 expression. *P<0.05 compared to control group.

tested in triplicates for average value. As shown in **Figure 2**, miR-126 expression had significant difference between patient and control groups. Patients had significantly higher miR-126 expression compared to control group (P=0.024). The relative expression of miR-126 was about 3.32 folds of that in control group.

qRT-PCR for DNMT1 expression

Samples from both groups were tested for average values. DNMT1 expression had significant difference between patient and control group. As shown in **Figure 3**, DNMT1 expression in patient groups was significantly lower than control group (P=0.031). Correlation analysis found that DNMT1 expression was negatively correlated with miR-126 expression (r=0.643, P=0.021). These results indicated that in peripheral T cells of RA patients, DNMT1 was under negative regulation of miR-126.

Table 2. Methylation level of EGFL7 and T cells

Group	EGFL7 (%)
Control	0.62±0.35
Patient	0.32±0.24
P value	0.032

EGFL7 methylation level

Sodium bisulfite sequencing results showed that methylation level of EGFL7 in patient group was significantly higher than that of control group (0.32±0.24 vs. 0.62±0.35, P<0.05). T cells in patient group showed hypo-methylation status (**Table 2**).

Cellular DNA methylation assay

DNA methylation level in T cells was measured by methylation specific AP-PCR approach. Results were shown in **Figure 4A**. PCR products were further tested in 1.5% agarose gel electrophoresis. Intensity of DNA bands in patient group was significantly lower than that of control group (grey value analysis was shown in **Figure 4B**), indicating relative hypo-methylation status in patient group compared to control ones.

Discussion

Elevation or decrease of DNA methylation level plays a critical role in regulating gene expression and genome integrity, and is the most common epigenetics modification form [12, 13]. Previous studies showed that DNA methylation was the common form to regulate gene expression. Abnormal methylation level of T cell genome has been demonstrated to be existed in multiple diseases. The level of methylation is closely correlated with occurrence of immune diseases such as SLE, tumor or RA [14, 15]. Respiratory failure is the major reason for death as the consequence of RA. As one autoimmune disorder, RA onset is correlated with change of

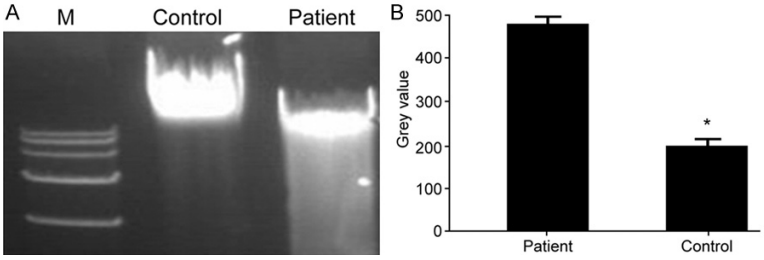

Figure 4. DNA AP-PCR results of T cells between two groups. A: Agarose gel electrophoresis for AP-PCR results; B: Analysis of electrophoresis bands grey values. *, statistical significance compared to control group.

T cell auto-immunity. Previous study showed hypo-methylation of T cells in RA patients. Therefore, the study of regulatory mechanism for such T cell hypomethylation in RA patients complicated with respiratory failure may provide molecular targets for gene therapy in clinics [16, 17].

miR participates in almost all functional gene expression regulation, and plays a critical role in gene translation and transcription, mostly negative control. Currently the study of miR has made major advancement, as it has been found to have important regulatory roles in multiple human diseases. The assay for specific miR has become marker for early tumor onset, whilst other miR has been recognized as the target for clinical treatment. Therefore, the study of correlation between miR and disease-related genes can explain pathogenesis mechanism from molecular level, and may provide valuable targets for drug treatment. Study has shown certain correlation between miR-126 and hypo-methylation level of T cells, which has host gene in EGFL7. The methylation level of EGLF7 is positively correlated with miR-126 expression [18-20]. DNMT1 is one sustained DNA methyl-transferase, and plays a critical role in methyl transfer.

Conclusion

This study showed hypomethylation in peripheral blood T cells in RA patients with respiratory failure. MiR-126 expression is negatively correlated with DNMT1 expression. In T cells of RA patients with respiratory failure, miR-126 expression is significantly lower than that of control group. Moreover, we found elevated methylation level of EGFL7 in peripheral T cells in RA patients with respiratory failure. In sum, we propose the possible mechanism inside peripheral T cells of RA patients with respiratory failure: elevated miR-126 expression further inhibits DNMT1 expression, causing the inhibition and DNMT1 activity, eventually leading to the inactivation of methyl inside T cells. Therefore, RA patients with respiratory failure have hypo-methylation in peripheral T cells. However, hyper-methylation of EGFL7 may further facilitate miR-126 up-regulation, which further accelerates hypo-methylation of T cells. This study demonstrated the role of miR-126 in hypomethylation in peripheral T cells of RA patients with respiratory failure. Gene regulation, however, is one complicated process. More studies are thus required to fully illustrate hypomethylation of peripheral T cell sin RA patients with respiratory failure.

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Disclosure of conflict of interest

None.

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