ORIGINAL ARTICLE

Differential expression analysis of miRNA in peripheral blood mononuclear cells of patients with non-segmental vitiligo

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ABSTRACT

Vitiligo is a common depigmentary skin disease that may follow a pattern of multifactorial inheritance. The essential factors of its immunopathogenesis is thought to be the selective destruction of melanocytes. As a new class of microregulators of gene expression, miRNA have been reported to play vital roles in autoimmune diseases, metabolic diseases and cancer. This study sought to characterize the different miRNA expression pattern in the peripheral blood mononuclear cells (PBMC) of patients with non-segmental vitiligo (NSV) and healthy individuals and to examine their direct responses to thymosin α1 (Tα1) treatment. The miRNA expression profile in the PBMC of patients with NSV was analyzed using Exiqon’s miRCURY LNA microRNA Array. The differentially expressed miRNA were validated by real-time quantitative polymerase chain reaction. We found that the expression levels of miR-224-3p and miR-4712-3p were upregulated, and miR-3940-5p was downregulated in the PBMC. The common clinical immune modulator Tα1 changed the miRNA expression profile. Our analysis showed that differentially expressed miRNA were associated with the mechanism of immune imbalance of vitiligo and that Tα1 could play an important role in changing the expression of these miRNA in the PBMC of patients with NSV. This study provided further evidence that miRNA may serve as novel drug targets for vitiligo therapeutic evaluation.

Key words: blood circulation, immune cells, miRNA, non-segmental vitiligo, peripheral blood mononuclear cells.

INTRODUCTION

Non-segmental vitiligo (NSV) is a common T-cell-mediated skin immune disorder characterized by the autoimmune destruction of melanocytes. The clinical manifestations are irregular patches of depigmentation involving the skin and mucous membranes. Studies of vitiligo patients have shown that the disease is often associated with other autoimmune diseases.1 Peripheral blood mononuclear cells (PBMC) consist of leukocytes in the blood circulation that have a single round nucleus such as lymphocytes (T cells, B cells), natural killer cells, monocytes and dendritic cells, and constitute a critical component of the immune system. The skin is a large and important peripheral lymphoid organ into which many immune cells migrate from blood circulation. Recent reports have shown infiltrated T-helper 17 cells in lesional skin and elevated interleukin-17 levels in the serum of patients with vitiligo vulgaris.2,3 These results indicated the importance of immune cells and secreted cytokines to vitiligo etiology.

The genetic information stored in DNA is utilized through an excellent management and coordination mechanism. A small RNA, called miRNA, exerts its function through adhering to the mRNA encoding sequence by inhibiting protein translation, a process that is quite common in mammals. Many in vitro animal models and experiments have shown that miRNA play important roles in the immune system, metabolic diseases and cancer, and are involved in common physiological responses to physical exercise.4,5 Several reports on the differential expression of host and viral miRNA and their roles in HIV infection were published recently.6,7 For example, Saif et al. recently reported for the first time that PBMC and plasma levels of miR-150 and miR-146b-5p are predictive of HIV/AIDS disease progression and response to antiretroviral therapy.8

As a modulator of immune response, thymosin α1 (Tα1) has been shown to possess multiple biological functions and maintain the balanced immune system.9–11 Understanding how Tα1 regulates gene expression at the level of miRNA will likely identify the mechanism of Tα1 injection as specific drug therapies.
The primary purpose of this study was to analyze the differential miRNA expression in the PBMC of patients with NSV for the first time. A second goal was to test the hypothesis that T≤1 alters the miRNA profile in the PBMC of patients.

METHODS

Subject information and sample collection
Thirty-two patients were enrolled from Qilu Hospital and Jinan Central Hospital, Shandong University. All patients were diagnosed with non-segmental vitiligo in progression using standard clinical diagnostic criteria and without systematic immune therapy for at least 1 month. Patients with segmental and localized types of vitiligo were excluded from participation. The white patch area was more than 3% of the body surface area.

Eighteen age- and sex-matched healthy individuals were also recruited. The study protocol was approved by each institution’s ethics committee, and written informed consent was obtained from all subjects. Fresh peripheral venous blood was extracted from subjects and treated with ethylenediaminetetra-acetic acid anticoagulants.

PBMC isolation and RNA extraction
Peripheral blood mononuclear cells were isolated from anticoagulated venous blood using Histopaque-1077 Density Gradient Centrifugation. miRNA in PBMC were extracted using Trizol Reagent (Invitrogen, San Diego, CA, USA) and the miRNeasy mini kit (QIAGEN, Hilden, Germany) from the isolated cells for microarray and real-time quantitative polymerase chain reaction (PCR) analysis.

miRNA expression profiling
After miRNA extraction from the 10 samples, expression profiling was performed using the 7th generation miRCURY LNA microRNA Array (version 18.0; Exiqon, Vedbaek, Denmark). The miRCURY Hy3/Hy5 Power labeling kit (Exiqon) was used according to the manual for miRNA labeling. One milligram of each sample was labeled and hybridized with the Hy3 fluorescent label. Following hybridization, the slides were scanned and imported into GenePix Pro 6.0 software (Axon) for data extraction. Replicated values were averaged and miRNA with intensities of 30 or more in all samples were chosen to calculate normalization factor. After normalization, significantly differentially expressed miRNA were identified through Volcano Plot filtering. Finally, hierarchical clustering was performed using MEV software (version 4.6; TIGR) to show distinguishable miRNA expression profiling among samples. The threshold of up- or downregulated screened miRNA was a fold change (FC) of 2.0 or more and P-value of less than 0.05.

Cell culture and T≤1 treatment
The peripheral blood monocytes isolated from patients were suspended in RPMI-1640 medium and then seeded in 12-well culture plates at 1 x 10⁶ cells/well. One milliliter of thymosin x1 (Zadaxin; SciClone, Foster City, CA, USA) was added to each well at 0, 50 or 100 µg/mL. PBMC were cultured with or without T≤1 for 72 h. After 72 h, the cells were collected by centrifugation, and miRNA were extracted for quantitative reverse transcription PCR (qRT-PCR) analysis.

miRNA real-time qRT–PCR
A stem-loop RT primer-based approach was used for cDNA synthesis. One microgram of total RNA was converted into first-strand cDNA using the TIANscript RT Kit (Tiangen Bio-tech, Tiangen, China). Total RNA was combined with the Bulge-Loop specific RT primer (RiboBio, Guangzhou, China) and U6 small nuclear RNA was used as the endogenous reference in each reaction.

Polymerase chain reaction amplification reactions were carried out with the KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Wilmington, MA, USA). A total volume of 20 µL per reaction contained 10 µL of KAPA SYBR FAST qPCR Master Mix (2×), 2 µL of template DNA, 1 µL of forward primer, 1 µL of reverse primer, 0.4 µL of ROX High and PCR-grade water. In the first cycle, reagent mixes were incubated at 95°C for 3 min, then 95°C for 3 s and finally 60°C for 30 s. All PCR reactions were performed on the ABI 7300 system (Applied Biosystems, Foster City, CA, USA). The specificity of reactions was determined by melting curve analysis. The relative expression levels of miRNA were calculated using the 2⁻ΔΔCT method. We also analyzed the relationship between the expression of miRNA by the LNA Array and by qRT–PCR technology by Pearson’s correlation analysis.

Statistical analysis
ANOVA and Student’s t-test were used to analyze the differences in all patients and healthy individuals. P-values of less than 0.05 were considered significant.

RESULTS

Clinical characteristics of patients with NSV
The clinical characteristics of patients with NSV are summarized in Table 1. A total of 32 patients including 19 sporadic vitiligo patients, nine generalized vitiligo patients and four acral vitiligo patients were enrolled. Of these patients, nine had been diagnosed with other autoimmune diseases and six had autoimmune thyroid disease.

Table 1. Clinical characteristics of 32 patients with vitiligo vulgaris

<table>
<thead>
<tr>
<th>Age range (years)</th>
<th>8–68</th>
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<tr>
<td>Sex (male : female)</td>
<td>15:17</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>0.1–25</td>
</tr>
<tr>
<td>Body surface area involved (%)</td>
<td>3–30</td>
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<tr>
<td>Subclassification of vitiligo patients’ sporadic vitiligo</td>
<td>19</td>
</tr>
<tr>
<td>Generalized vitiligo</td>
<td>9</td>
</tr>
<tr>
<td>Acral vitiligo</td>
<td>4</td>
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Differentially expressed miRNA screening in patients with NSV and healthy individuals

To explore the potential role of miRNA in the mechanism of the immune imbalance involved in vitiligo, we analyzed the miRNA expression data in PBMC from non-segmental vitiligo patients ($n = 5$) and healthy individuals ($n = 5$) using miRNA LNA Arrays. We identified four miRNA that were significantly differentially expressed in the PBMC of patients with NSV. One miRNA was downregulated (miR-3940-5p, FC = 0.43, $P = 0.03$) and three miRNA were upregulated (miR-224-3p, FC = 4.71, $P = 0.04$; miR-2682-3p, FC = 3.69, $P = 0.01$; miR-4712-3p, FC = 2.55, $P = 0.002$) relative to healthy controls (Fig. 1a).

To confirm the miRNA array expression findings, stem-loop reverse transcription followed by a SYBR Green PCR assay was carried out to verify the expression level of the four miRNA in a larger patient population. We analyzed the expression levels of four miRNA in the PBMC of patients with NSV ($n = 32$) and from healthy controls ($n = 18$). Consistent with the microarray data, we found that the expression levels of miR-224-3p, miR-4712-3p were upregulated and miR-3940-5p was downregulated in the PBMC (Fig. 1b, $P < 0.05$), but there was no significant difference in the upregulated expression level of miR-2682-3p as detected by PCR ($P > 0.05$).

This analysis showed that the expression of four miRNA determined by LNA Array technology positively correlated with the qRT-PCR results. As shown in Table 2, the fold change of three miRNA was highly correlated ($r = 1.000$, $P = 0.005$) between two methods.

Effect of Tα1 treatment on the expression of miRNA in PBMC

To obtain a more detailed understanding of the function of Tα1 on immune cells of blood circulation, we cultured PBMC of patients with or without Tα1 and observed whether Tα1 regulated the expression levels of miRNA in immune cells. Under the microscope scattered, suspended distribution, translucent, round cells were observed. After treatment for 72 h, the number and aggregation of cells increased. We also examined the expression levels of four miRNA in PBMC by qRT-PCR and found that the levels of miR-224-3p, miR-2682-3p and miR-4712-3p in cultured PBMC were lower and miR-3940-5p was higher in the presence of Tα1 compared with the groups without Tα1, but there was no statistical significance between the two concentrations (Fig. 2). The results suggested that Tα1 may modulate the immune response via miRNA.

Figure 1. Differential expression of miRNA in peripheral blood mononuclear cells (PBMC) from vitiligo patients compared with healthy individuals. (a) Heat map showing the miRNA array data from patients ($n = 5$) and healthy controls ($n = 5$). (b) Real-time quantitative polymerase chain reaction analysis showing differentially expressed miRNA in PBMC from patients ($n = 32$) and healthy controls ($n = 18$). *$P < 0.05$. © 2014 Japanese Dermatological Association
miRNA are a class of single-stranded non-coding small RNA that regulate gene expression in numerous cellular processes including development, differentiation, proliferation, apoptosis and stress response. A recent study provided the first comprehensive analyses of serum miRNA expression profiles in patients with NSV and discovered that the miR-16, miR-19b and miR-720 appeared to be the best serum biomarkers to distinguish NSV cases from healthy controls.14 Mansuri et al.15 identified differentially expressed miRNA in skin lesions of non-segmental vitiligo. In this study, compared with healthy controls, the miRNA expression data in PBMC from vitiligo patients was analyzed using a high-throughput miRNA array platform that was able to assess the expression of almost 3000 human miRNA and viral miRNA.

We first identified four differentially expressed miRNA from patients with NSV, including one downregulated miRNA and three upregulated miRNA. We further validated the results using qRT–PCR. Available qRT–PCR technology can easily detect low-abundance circulating miRNA. The extraordinary stability of miRNA and the relative convenience of their extraction, quantification and detection through PCR technology make miRNA attractive candidates for biomarker selection.16

We found that some of the altered miRNA are related to immune and cancer cells. The expression of miR-224-3p was increased during the innate immune response in mouse lung following exposure to aerosolized lipopolysaccharide and correlated with a reduction in the expression of tumor necrosis factor-α, keratinocyte-derived chemokine and macrophage inflammatory protein-2, suggesting a potential role for miRNA in the regulation of inflammatory cytokine production.17 miR-3940-5p was significantly downregulated in non-small cell lung carcinoma tumor tissues and embryonic lung tissues compared with normal lung tissues which suggested that miR-3940-5p may be involved in the differentiation of stem cells of normal or cancer cells.18

Our data revealed that PBMC and miRNA are sensitive to immunomodulatory treatment, as demonstrated by growth state of cultured cells and the miRNA expression level in Tx1 treatment tests. Despite the significant experimental results, insufficient sample size and lack of observation of dynamic changes during vitiligo treatment are limitations of the current study. Further validation in a large cohort of vitiligo subjects and therapeutic evaluation in terms of miRNA changes are essential to future studies.

In summary, our study is the first to comprehensively analyze the miRNA expression profiles of global immune cells in blood circulation in patients with NSV. The data raise the possibility that miRNA may be involved in the development of non-segmental vitiligo. Based on these results, we have reason to believe that the specific miRNA signatures in PBMC become part of the vitiligo-associated immune response, and miRNA may serve as novel drug targets for vitiligo therapy.

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CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

REFERENCES


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