MicroRNA expression profiling of peripheral blood mononuclear cells associated with syphilis

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Research article

Keywords: Syphilis, microRNA profiling, peripheral blood mononuclear cells, Treponema pallidum, sexually transmitted infections

Posted Date: May 31st, 2019

DOI: https://doi.org/10.21203/rs.2.9964/v1

Version of Record: A version of this preprint was published on February 22nd, 2020. See the published version at https://doi.org/10.1186/s12879-020-4846-x.

Abstract

Background Treponema pallidum (T. pallidum Tp) infection evokes vigorous immune responses, resulting in tissue damage. The immune mechanism after Treponema pallidum infection is still not clear. MicroRNAs (miRNAs) have been shown, however, to influence immune cell function and consequently the generation of antibody responses during other microbe infections, but these values are unknown for Tp. Methods In this study, we performed a comprehensive analysis of differentially expressed miRNAs in healthy persons, untreated patients with syphilis, patients in the serofast state, and serologically cured patients. MiRNAs were profiled from patient peripheral blood obtained at the time of serological diagnosis. Then both the target sequence analysis on these different miRNAs and pathway analysis were performed to identify important immune and cell signaling pathways. Quantitative RT-PCR for analysis of microRNA Results There were 89 differentially regulated miRNA identified in total. Following RT-qPCR confirmation, three miRNAs (hsa-miR-195-5p, hsa-miR-223-3p, hsa-miR-589-3p) showed significant difference among serofast state, and serological cure ($P<0.05$). A miRNAs (hsa-miR-195-5p) showed significant differences among untreated patients and healthy individuals. Conclusions This is the first study of miRNA expression difference in PBMCs in different stages of T. pallium infection. Our study suggests that the combination of three miRNAs has great potential to serve as non-invasive biomarkers of Treponema pallidum infections, which will facilitate better diagnosis and treat of T. pallium infections.

Background

Syphilis is caused by infection with the spirochete, *Treponema pallidum* subsp. pallidum (*T. pallidum*) [1,2]. It is one of the most common sexually transmitted diseases worldwide. Syphilis is a multistage progressive disease with a variety of manifestations, including chancre, disseminated skin lesions, gummas, neurosyphilis, and cardiovascular syphilis [2]. Syphilis symptoms usually resolve with appropriate antibiotic medications. However, the evaluation of therapeutic response depends on serological testing. Patients with nontreponemal titers that decline 4-fold or more are considered as having a good serological response, whereas those with neither increase nor decrease 4-fold are referred to as being “serofast” [3]. The proportion of serofast patients has reached 30–40%[3]. It is not known why symptoms and severity vary so greatly in syphilis patients, or why serological reactions are not mitigated in serofast patients, but is likely the outcome of host immune responses elicited by *T. pallidum*. *Treponema pallidum* cannot be cultured in vitro, so the pathogenesis of syphilis is not yet clear. The current diagnostic methods for syphilis cannot distinguish between serofast state and latent syphilis. Many researchers are currently attempting to develop new biomarkers to help treat this disease.

Macrophages have been shown to be activated during syphilis infection, as proved by the production of macrophage-activating factors (MAFs) from syphilitic rabbits [4]. Dendritic cells (DCs), which are the most potent antigen-presenting cells, dendritic cells (DC) can phagocytize *T. pallidum* and produce inflammatory cytokines, including interleukin 1β (IL-1β), IL-6, and tumor necrosis factor alpha (TNF-α) [5], which are crucial for the initiation of T cell responses to *T. pallidum* infection. Evidence of T lymphocytes infiltrating syphilis lesions was provided by Engelkens et al. [6]. Previous studies have

demonstrated that the Th1 cytokines IL-2, IL-12, and gamma interferon (IFN-γ) were predominantly expressed by both the infiltrating T cells in lesions [7] and splenic lymphocytes stimulated by sonicated *T. pallidum* [8]. However, the Th1 response is suppressed by Th2 cytokine IL-10 with the progression to latent syphilis [9], which shows a strong Th2-mediated humoral immune response. Immunosuppression also takes place in syphilitic serofast patients, with the evidence of obviously increasing regulatory T cells (Treg) which have potent immunosuppressive activity [10]. However, the mechanism underlying immune regulation in syphilis infection remains unclear. The abnormalities of immune cells in syphilis were induced by a complex way involving genomic and transcriptomic changes. Many studies have established that pathogens can affect host immunity by regulating host microRNA expression.

MicroRNAs (miRNAs) are evolutionarily conserved small noncoding RNA molecules. The sequence of microRNAs usually includes 19–24 nucleotides. They can bind to the target mRNA, resulting in translational suppression or degradation of mRNA [11]. Due to the important function of microRNAs, they regulate about 30% gene transcription involving in a variety of cellular processes, including the immune response to invading pathogens [12]. The miRNAs (i.e., miR-223-3p, miR-150, miR-146b, miR-16, and miR-191), abundantly expressed in T cells, were down regulated in HIV patients [13]. Moreover, HIV-1 gp120 could activate the STAT3 signaling pathway to regulate the expression of miRNA-21, miRNA-155, and miRNA-181b in monocyte-derived dendritic cells (MDDCs) [14]. Six serum miRNAs (miRNA-378, miRNA-483-5p, miRNA-22, miRNA-29c, miRNA-101, and miRNA-320b) have been reported to be differentially expressed in tuberculosis, which regulated some target genes associated with mitogen activated protein kinases (MAPK) and TGF-β signaling [15]. Altered expression of miRNA-155, related to immune activation and inflammation, was found to be regulated by IL-10, so playing an important role in suppression of *Borrelia burgdorferi*-induced Lyme arthritis and carditis [16]. These studies highlighted the importance of altered miRNAs in the immune response of infectious diseases. However, the study of miRNA profiles in syphilis is still limited.

This is the first study of miRNA expression difference in PBMC in different stages of *T. pallium* infection. In our present study, the data show that miRNA levels in serum increase after *T. pallium* infection, which verified miRNA-19b-3p was downregulated and related to suppression of Th1 production in syphilis [17]. In this work, we aimed to use microarray analysis to detect differential miRNA expression in peripheral blood mononuclear cells (PBMC) from syphilis patients, so attempting to identify the key molecule in dysfunctional immune cells. It may be useful to present novel biomarkers for syphilis diagnosis and prognosis.

Methods

1. Sample collection

Peripheral blood samples were obtained from individuals who visited the Dermatology Hospital of Southern Medical University, Guangzhou Panyu Center for Chronic Disease Control, Zhuhai Center for Chronic Disease Control, Yingde Center for Chronic Disease Control and Shenzhen Nanshan Center for

Chronic Disease Control. All samples were diagnosed using RPR and TPPA. According to Chinese syphilis prevention and treatment guideline (version 2015), serofast state was defined as infected patients with positive *T. pallidum* particle agglutination assay (TPPA) and a low positive toluidine red unheated serum regain test (RPR) lasted for 1 year after standard treatment. Serological cure was defined as positive TPPA and negative RPR.

Exclusion criteria were as follows: 1) Co-infected with human immunodeficiency virus, condyloma acuminata, and other sexually transmitted disease. 2) Patients suffering from autoimmune disease, undergoing anti-inflammatory or immunosuppressive therapy, or who have taken antibiotics within the past 6 months.

This study was approved by the Ethics Committee at the Guangdong Provincial Dermatology Hospital. The objectives, procedures, and potential risks were verbally explained to all participants. Written informed consent was obtained from all patients prior to inclusion in this study.

2. RNA extraction (Ref: Int. J. Mol. Sci, 2017, 18, 1471)

Peripheral blood mononuclear cells (PBMC) were isolated from the whole blood by a standard procedure of Ficoll gradient centrifugation, performed in strict accordance with the manufacturer's instructions. Total RNA in PBMCs was extracted using Trizol reagent. RNA was quantified using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, US). RNA was suspended in RNase free water and stored at -80°C.

3. MicroRNA expression profiling by microarray (Infect Genet Evol, 2017, 54, 152-157)

As table 1 shows six heathy volunteers, 6 syphilis patients before treatment, 6 syphilis patients in the serofast state, and 6 serologically cured patients were enrolled. Human miRNA OneArray® v5.1 (Phalanx Biotech Group, Taiwan) contains triplicate 2,539 unique miRNA probes from Human (miRBase Release 20), and 114 experimental control probes. The detailed descriptions of the gene array list are available from http://www.phalanx.com.tw/products/HmiOA_Probe.php#.

4. MicroRNA-predicted target genes and gene ontology (GO) and pathway analyses (Ref: Clinical Epigenetics, 2017, 9, 79)

Potential targeting miRNAs were predicted and analyzed using bioinformatics algorithms (miRWalk, DIANA-microT4, miRanda, miRDB, PICTAR2, and TargetScan) with miRWalk2.0 [21]. To reduce the number of false positives, only target genes that were predicted by at least four of the six programs were

selected and used for further investigation. The biological annotation and the potential pathway were analyzed using DAVID, version 6.7, and KEGG pathway enrichment analysis, respectively.

5. Quantitative RT-PCR for analysis of microRNA (Ref: Int. J. Mol. Sci, 2017, 18, 1471, Ref: Clinical Epigenetics, 2017, 9, 79)

We evaluated, 12 healthy controls and 94 syphilis patients using RT-PCR to verify the changes in miRNA expression using quantitative RT-PCR. A total of 49 current syphilis infection patients had not been treated with antibiotics before enrollment, including primary (n=14), secondary (n=18), early latent (n=10), and late latent syphilis (n=7) infections. In addition, 45 patients after standard treatment were divided into two groups: serofast state (n=28) and serological cure (n=17). All clinical information is listed in Table 2. Quantitative RT-PCR (RT-qPCR) analysis was performed using Mir-X miRNA RT-qPCR SYBR Kit (Takara Biomedicals, Shiga, Japan) according to the manufacturer's instructions. Briefly, each 1 μg of total RNA was reverse-transcribed to cDNA using the Mir-X miRNA First-Ssrand Synthesis Kit (Takara Biomedicals, Shiga, Japan). MicroRNA expression was analyzed in duplicate and normalized to U6 on a LightCycler 480 (Roche Applied Science, Basel, Switzerland). The fold change for microRNA was calculated using the comparative-Ct (△Ct) method.

6. Statistics

Statistical analyses were performed using GraphPad Prism software 5.01. Data are presented as mean ± standard deviation (SD) if not otherwise stated. The student's t-test was used for two groups comparison. Significance in microarray analysis among multiple groups was determined by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. P value < 0.05 was considered statistically significant.

Results

1. Sample QC and Principal Component Analysis

As a means of quality control to assess the biological separation of the groups based on probe intensities, principal component analysis (PCA) was performed using Partek Genomics software. Four separate groups of samples from heathy volunteers, syphilis patients before treatment, serofast state syphilis patients, and serologically cured syphilis patients are clearly observed (Figure 1). The principal component of serological cure group was far from serofast state. The principal components of different groups were different. D4 is very different from the group of serological cure patients. B2 is very different from the group of serofast state. C3 is also different from the group of syphilis patients before treatment.

2. Differentially Expressed miRNAs

We performed differential miRNA assays in the PBMCs of 6 uninfected samples from patients with syphilis, 6 untreated samples from patients with syphilis (3 of primary syphilis and 3 of secondary syphilis), 6 serofast samples, and 6 serological cure samples. Compared with those without infection samples, 42 miRNAs were up-regulated and 16 miRNAs were down-regulated in untreated patients with syphilis. Meanwhile, 15 miRNAs were up-regulated and one miRNA was down-regulated in serofast patients compared with serologically cured patients.

3. Differential gene target sequence prediction and pathway enrichment analysis

To understand the possible functions of these differential miRNAs, target sequence of differentiated miRNAs among the groups were predicted using the Targetcan database. The target genes were then subjected to KEGG pathway enrichment analysis. As shown in Fig. 2, there are several possible regulatory pathways of 18 discrepant miRNAs that meet the minimum P value. These pathways were the Mucin type O-Glycan biosynthesis, proteoglycans in cancer, endocytosis, adherens junction, pathways in cancer, signaling pathways regulating pluripotency of stem cells, GABAergic synapse, fatty acid biosynthesis, thyroid hormone signaling pathway, adrenergic signaling in cardiomyocytes, morphine addiction, phosphatidylinositol signaling system, arrhythmogenic right ventricular cardiomyopathy (ARVC), pancreatic cancer, glioma, Ras signaling pathway, and axon guidance pathways.

4. Bioinformatics network analysis of candidate miRNAs and corresponding target sequences

To understand the role of miRNAs and corresponding target genes during syphilis infection, we conducted an interplay analysis between miRNAs and corresponding target genes. The results could help us better explain the key regulatory functions of miRNAs. Fig. 3 shows 10 pathways with the lowest P value (Fig. 3) (most relevant) of discrepant miRNAs and corresponding target genes. Among them, the miRNAs of 497 target genes showed differences between syphilis patients and healthy individuals. There were 30 up-regulated miRNAs and 16 down-regulated miRNAs (Fig. 4A). And 213 target genes showed differences in miRNAs expression between serofast patients and serologically cured patients. There were 15 up-regulated miRNAs and 1 down-regulated miRNA (Fig. 4B).

5. The expression of 8 miRNAs in clinical specimens

Eight differentially expressed miRNAs were selected. In order to verify the relationship between these miRNAs and syphilis, 110 clinical specimens were used to detect the differential expression of miRNAs. The clinical information of all specimens is summarized in Table 2. Fig 5 shows the expression of 8 miRNAs in each group. The expression of miR-195-5p in the serofast state was significantly higher than in the serologically cured state and in healthy persons. It was also higher in primary syphilis than in healthy persons. There was no significant difference between the other groups. In addition, the expression of miR-223-3p and miR-589-3p in serofast state was significantly higher than in serological cure(Fig5 A). In this data, we can find that there is no significant difference between early latent syphilis and late latent

syphilis among most miRNAs. And there was no significant difference in the expression of other miRNAs. Furthermore, PBMCs of Healthy persons were incubated with *T.pallidum*. The data showed that miR-195-3p was up-regulated and miR-6870-3p was down-regulated after *T.pallidum* incubated (Fig5 B).

These results suggested that miR-195-5p, miR-589-3p and miR-223-3p may be related to the pathogenesis of serofast state. Meanwhile, miR-195-5p had a certain relationship with T.pallidum infection.

Discussion

T. pallidum remains one of human pathogens that cannot be cultivated in vitro to-date. There is also no suitable animal model for studying the pathogenesis of syphilis. These obstacles have greatly hindered the effort of elucidating the basic immunobiological traits of syphilis. *T. pallidum*-specific antibodies do not cause any significant change after treatment and have no detectable protective effect. Patients in the serofast state and patients with latent syphilis show about the same serological response. Serological diagnosis cannot distinguish the serofast state from latent syphilis, and the serofast state is not uncommon clinically. There is no evidence that serofast states are relevant to *Treponema pallidum*. Therefore, how to treat and identify serofast state is a very important issue for the prevention of syphilis. At the same time, the immune mechanism of syphilis is not clear. Several studies have shown that immunosuppression can occur after infection with *T. pallidum*. However, the mechanism of immune regulation is not clear.

Recently, miRNAs have been intensively studied as new biomarkers for diagnosis and prognosis in various diseases such as, cancers, heart disease, diabetes, psychosis, and infectious diseases [18-22]. Many researchers studying the expression profile of infectious diseases show that microRNAs play an important role in the host's anti-infective immune response. This study provides the first miRNA expression profile of peripheral blood samples from healthy individuals, untreated syphilis patients, patients in the serofast state, and serologically cured patients. In the present study[17], we used microarray analysis to assess the differential serum miRNA expression profile in syphilis patients and matched healthy controls. Among the differentially expressed microRNAs identified by microarray analysis, miR-21-5p, miR-19b-3p, miR-16-5p, and miR-142-3p were selected as candidates for further testing using RTq-PCR.

In this study, we found 89 differentially expressed miRNAs. According to the microarray analysis, forty-two miRNAs in untreated syphilis patients were up-regulated relative to healthy individuals, and sixteen miRNAs were down-regulates. Fifteen miRNAs in the serofast state were up-regulated relative to serologically cured patients, and a miRNA was down-regulated. However, we did not find the same differential miRNAs upon examining serum. There were many miRNAs expressed in untreated syphilis patients than in healthy controls, indicating that *T. pallidum* infection can lead to changes in immune mechanisms.

miRNAs regulate gene expression by binding to complementary site on mRNAs and reducing mRNA stability and translation [23]. miRNAs could regulate gene expression and work as transcription factors by regulating development timing and differentiation of cells. Alteration in miRNA expression may have affected the signaling pathway. Pathway analysis helped us to assess the biological processes in immune responses of miRNAs and target genes. In this study, pathway analysis indicated that predicted target genes for those miRNAs were involved in the mucin type O-glycan biosynthesis, proteoglycans in cancer, endocytosis, adherens junction, pathways in cancer, signaling pathways regulating pluripotency of stem cells, GABAergic synapse, fatty acid biosynthesis, thyroid hormone signaling pathway, adrenergic signaling in cardiomyocytes, morphine addiction, phosphatidylinositol signaling system, arrhythmogenic right ventricular cardiomyopathy (ARVC), pancreatic cancer, glioma, Ras signaling pathway, and axon guidance pathways. Adherence-mediated colonization plays an important role in pathogenesis of microbial infections, particularly those caused by extracellular pathogens responsible for systemic diseases, such as *Treponema pallidum* subsp. pallidum, the agent of syphilis. Many studies have shown that outer membrane proteins of *T. pallidum*, such as TP0136, TP0155, and TP0483. could participate in the adhesion mechanism [24,25]. Natural immunity is the first barrier after *T. pallidum* infection. A variety of lipoproteins of *T. pallidum* activate phagocytes and dendritic cells (DCs) through CD14, toll-like receptor 1 (TLR1) and TLR2-dependent signaling pathways, and these pathogen-associated pattern molecules (PAMPs) are thought to the main pro-inflammatory factors in the process of *T. pallidum* infection. Special outer membrane structures of *T. pallidum* that lack exposed surface lipoprotein cause PAMPs to act as refractory to TLRs or other pattern recognition receptors (PRRs) of macrophages or DCs. In this way, innate immunity cannot be activated, and *T. pallidum* cannot be cleared by the immune system [26]. The miRNAs of these signaling pathways may be suitable targets for research into syphilis immunomodulation.

The miRNA-gene-network helped us to screen miRNAs important to regulating immune response. In this study, miRNAs might play an important role in the regulation of syphilis immune mechanism, such as miR-195-5p and miR-223. We used RTq-PCR to verify the expression of different miRNAs, significant difference was found in the expression of three miRNAs (hsa-miR-195-5p, hsa-miR-223-3p, hsa-miR-589-3p) of PMBCs of 106 samples. First, we are interested in miR-195-5p. Most studies of miR-195-5p focus on the regulatory role of apoptosis, which could inhibit the expression of some anti-apoptotic proteins [27-30]. One recent study showed that miR-195-5p can inhibit macrophages' proinflammatory expression [31]. Interestingly miR-195-5p were here found to be differentially expressed between the serofast state and latent syphilis (all of early latent and late latent syphilis, data not shown). Furthermore, miR-195-5p was up-regulated after *T.pallidum* incubated in PBMCs of Healthy persons. These data suggest that miRNAs are associated with *T.pallidum* infection. In addition, miRNA-223-3p can inhibit the proinflammatory responses in Helicobacter pylori-infection macrophages. Macrophage-mediated inflammation can activate cellular immunity. We suppose that it affects the syphilis immune mechanism by affecting the apoptosis of immune cells, and it is involved in the regulation of syphilis inflammation.

The causes of the serofast state are the subject of some debate. Some studies maintain it is due to *T. pallidum* not being completely eliminated. However, there is no evidence that *T. pallidum* is latent. RT-

qPCR data indicate that individual differences in serological cure are small. However, individual differences in the serofast state are more pronounced. We think this is due to the unclear definition of serofast state. Results of serological testing of the serofast state are very similar to those of latent syphilis. In this way, we find the serofast state to be a complicated problem. Studying the regulation of miRNAs on immunity can improve our understanding of the serofast state.

In summary, our results may have suggested that changes in miRNAs expression profiles may be associated with immune tolerance and *T. pallidum* persistence infection through regulation of target genes or signaling pathways. miRNAs that are differentially expressed among syphilis patients, such as miRNA-195-5p, could be new biomarkers for *T. pallidum* persistence. They might be a good candidates for investigation of the mechanism for *T. pallidum* clearance. Blocked or decreased expression of these miRNAs might have some role in the treatment of syphilis and management of the serofast state.

Declarations

Funding:

This work was supported by grants from bellows:

1. The National Natural Science Foundation of China (NSFC) 81772240
2. Guangdong Science and Technology Department 2017A020212008
3. Medical Science and Technology Foundation of Guangdong Province C2017043
4. Medical Science and Technology Foundation of Guangdong Province A2016269
5. Medical Science and Technology Foundation of Guangdong Province A2016239

Conflict of Interest:

The authors declares that have no competing interests related to the content of the specific article.

Ethical approval:

Ethical approval was obtained from the ethics committee of Dermatology Hospital, Southern

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Tables

Due to technical limitations, the tables have been placed in the Supplementary Files section.

Figures

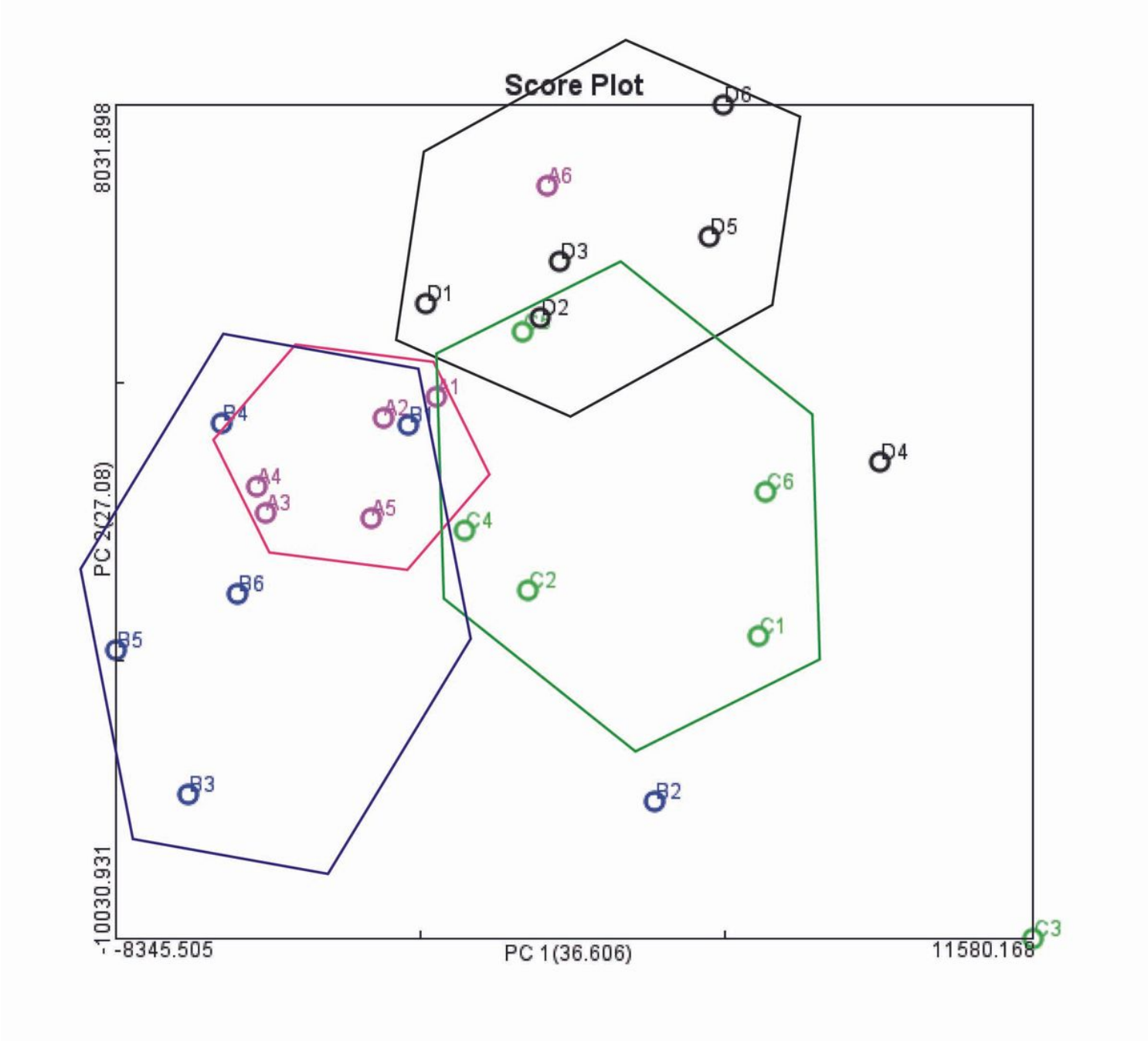

Figure 1

Principal component analysis (PCA) on miRNA expression data from human peripheral blood mononuclear cells (PBMCs). Red, A1-A6, healthy individuals; blue, B1-B6, serofast state; green, C1-C6, syphilis patients before treatment; black, D1-D6, Serological cure.

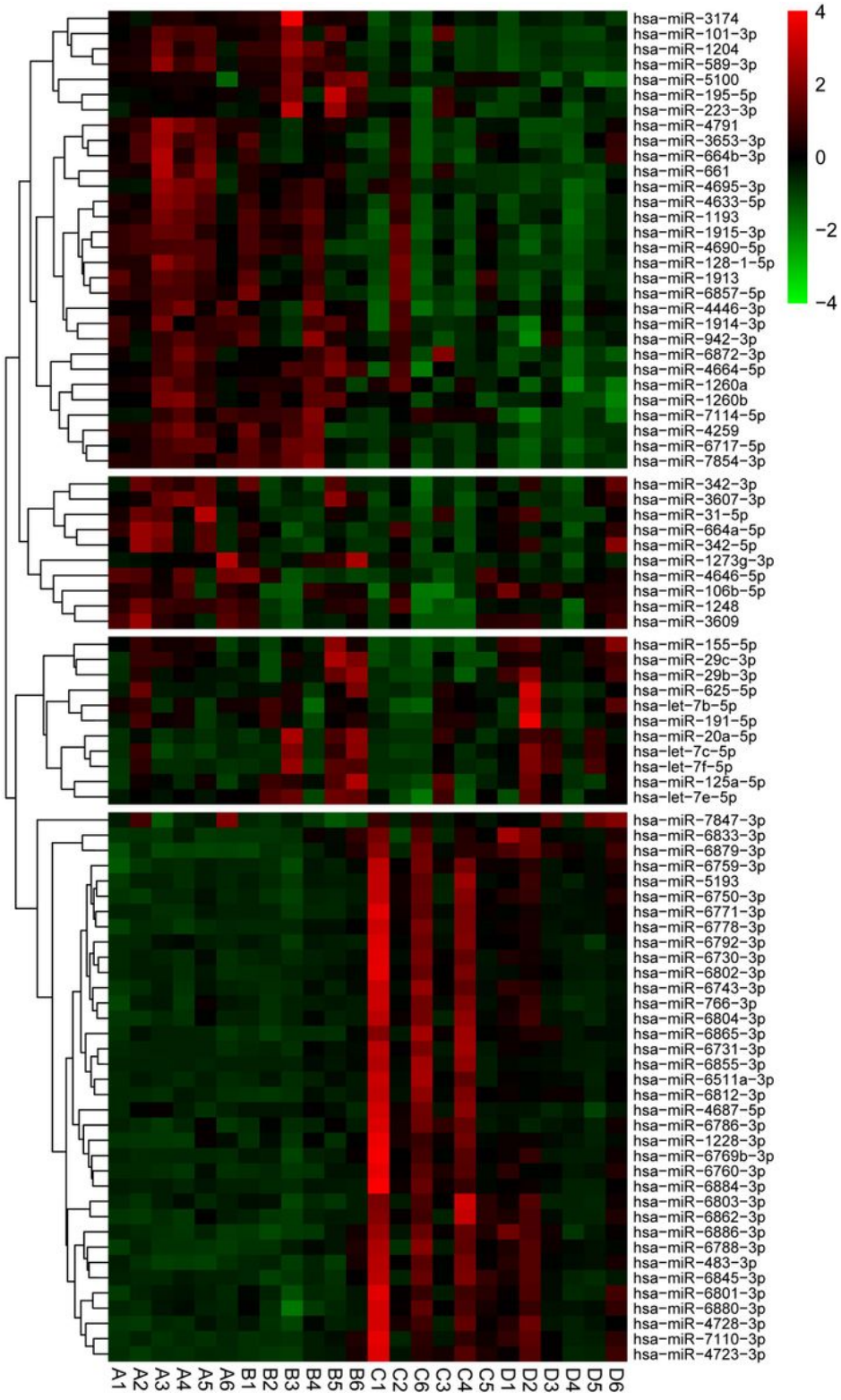

Figure 2

miRNA screening in syphilis patients, healthy individuals, serofast patients, and serologically cured patients. Red boxes indicate up-regulated miRNAs, and green boxes indicate down-regulated miRNAs. The brightness indicates the magnitude of the difference. Changes in miRNAs expression N ≥1.5-fold, and Q<0.05 are illustrated by the heat map. For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article.

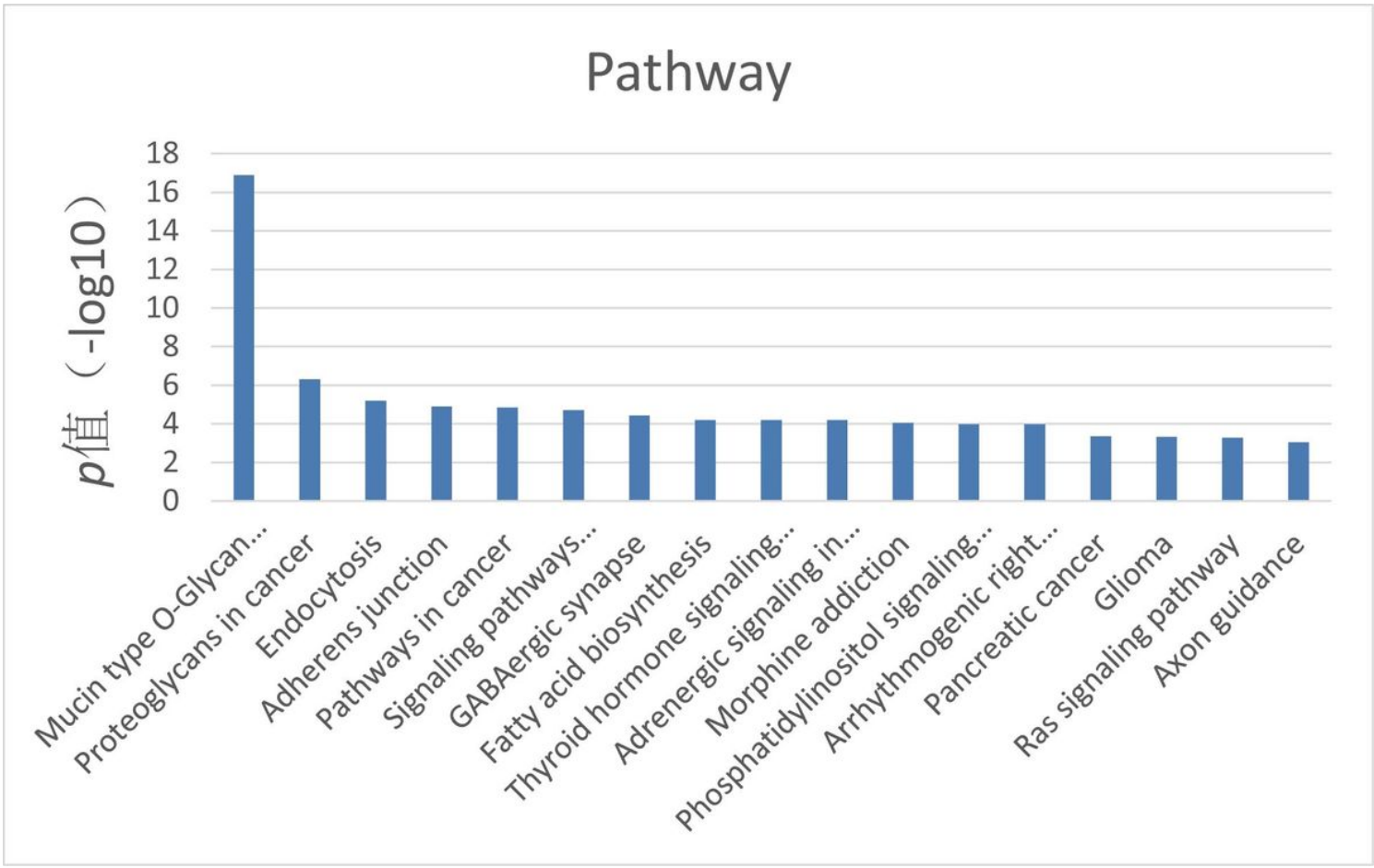

Figure 3

Pathway analysis. The top 18 significantly changed pathways for target genes. The Y-axis shows negative logarithm of P value (−lg p), and blue bars shows the changed pathways.

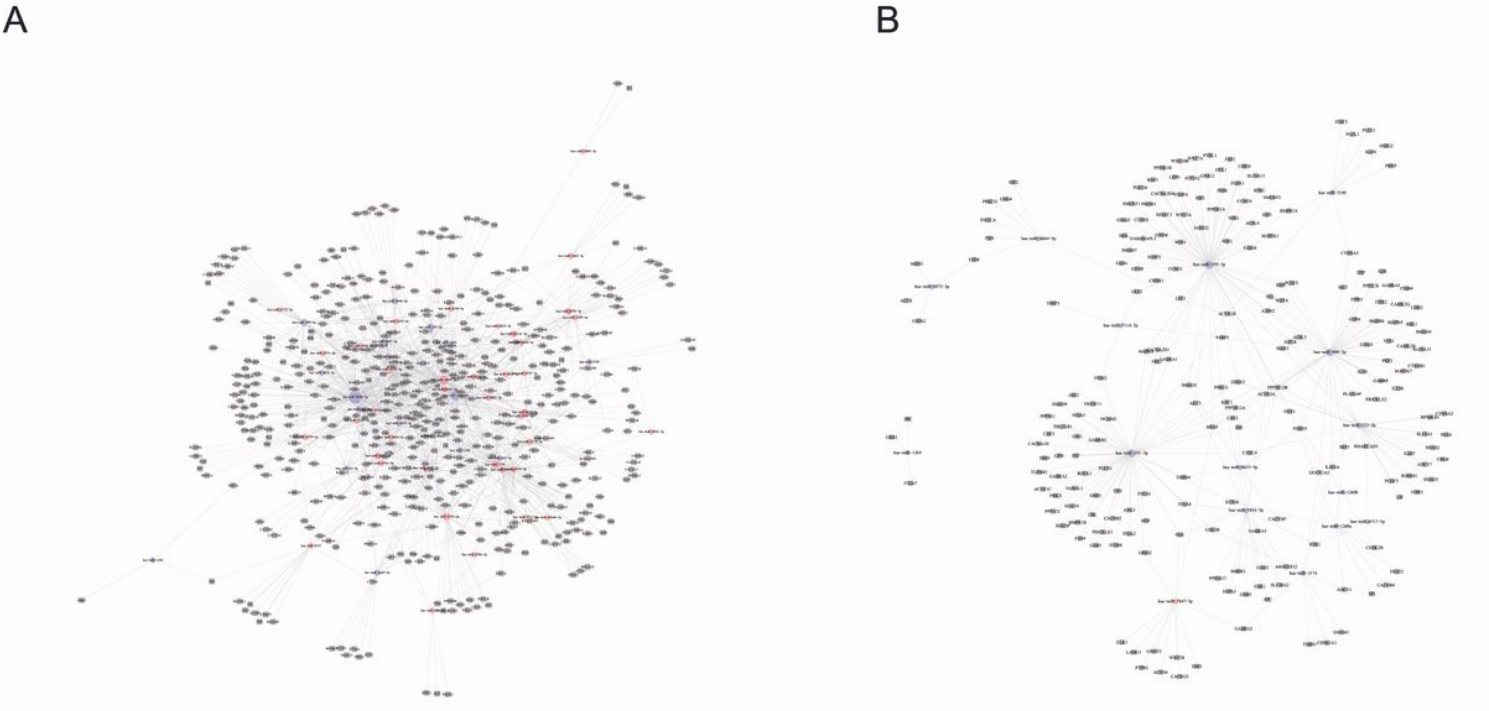

Figure 4

MicroRNA-gene-network. MicroRNA-gene-network showed us the predicted target genes were regulated by miRNAs. Square grid nodes represented microRNA, cycle nodes represented target genes, red means up-modulated and blue means down-regulated. The size of the circle or square represented the degree value. The bigger one indicated the miRNA play more critical role in regulation. (a) Healthy persons and syphilis patients; (b) serofast patients and serologically cured patients.

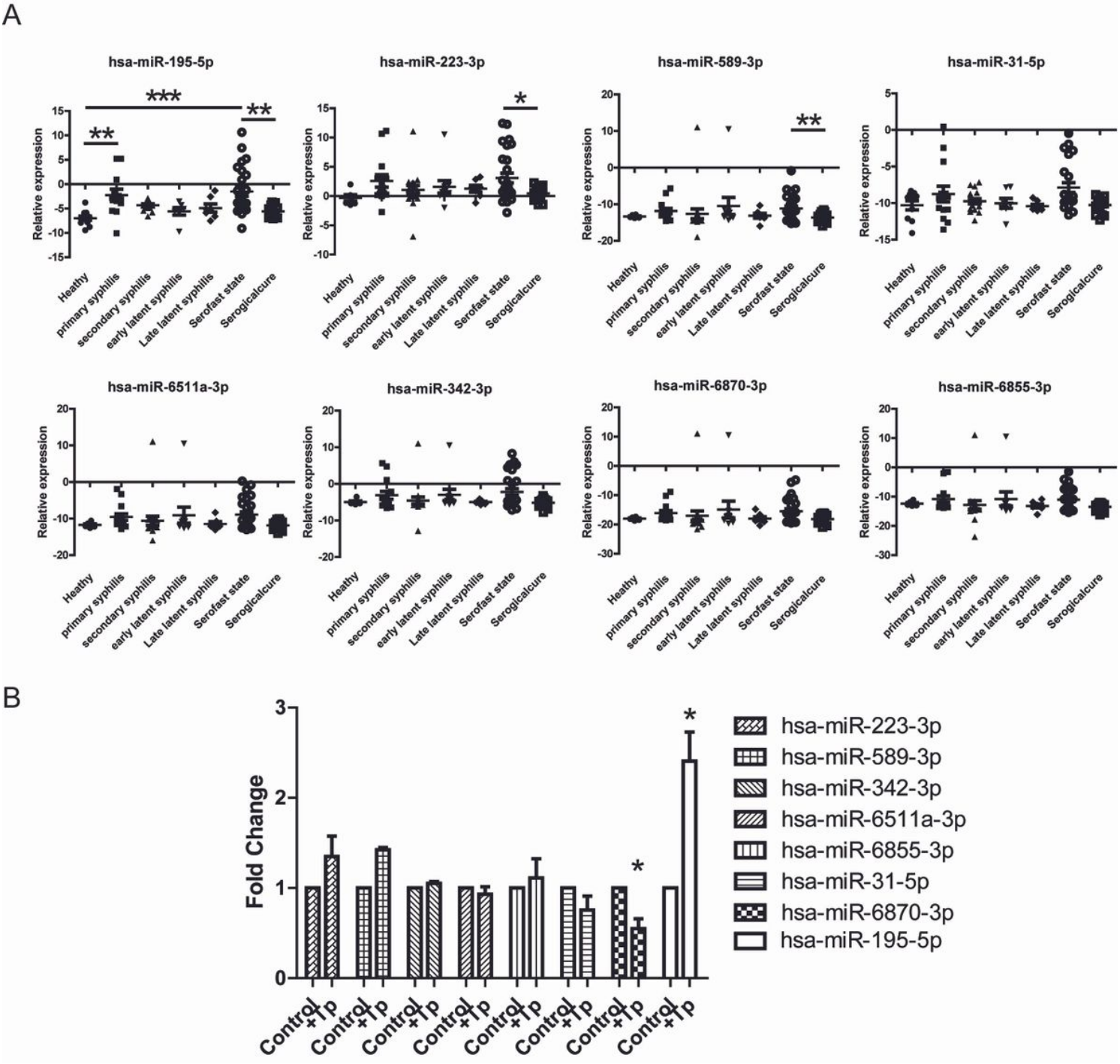

Figure 5

A) Detection of pulmonary PBMCs of different stages miRNAs by RTq-PCR assay. The expression of 8 miRNAs was measured in 106 samples. We analyzed the expression of 8 miRNAs (hsa-miR-195-5p, hsa-miR-223-3p, hsa-miR-589-3p, hsa-miR-342-3p, hsa-miR-6511a-3p, hsa-miR-6870-3p, hsa-miR-31-5p, hsa-miR-6855-3p) selected from the microarray data by using RT-PCR. Relative expression was used to normalize the relative gene expression data in the RT-qPCR assay. U6 was set as the reference gene. Statistical analysis was performed using the nonparametric Mann-Whitney test. ***P<0.001, **P<0.01, *P<0.05. B) PBMCs of Healthy persons were incubated with T.pallidum. And the expression of 8 miRNAs was measured by RT-PCR.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.tif
- supplement2.tif