Peripheral blood mononuclear cell microRNA profiles in syphilitic patients with serofast status

Xinmiao Jia1 · Zhongshuai Wang2 · Xiaoke Liu2 · Heyi Zheng2 · Jun Li2

Received: 15 September 2019 / Accepted: 3 April 2020 / Published online: 24 April 2020
© Springer Nature B.V. 2020

Abstract
Syphilis is a chronic sexually transmitted disease caused by infection with *Treponema pallidum*, which can invade various system organs, leading to clinical manifestations such as neurosyphilis, ocular syphilis, and cardiovascular syphilis and seriously endangering human health. Serofast status is a common outcome after syphilis treatment that presents an important clinical problem. At present, the etiology of serofast status remains unknown. A systematic investigation of the microRNA (miRNA) expression profiles in peripheral blood mononuclear cells (PBMCs) of patients with serofast status or secondary syphilis and of healthy control subjects was conducted using small RNA-seq. The expression of miRNAs was further confirmed by real-time fluorescence quantitative PCR (qPCR) assays. The data reveal a specific miRNA expression profile that was displayed in cells from patients with serofast status. Known and novel predicted (np)-miRNAs were also identified and verified, such as miR-338-5p, np-miR-163, np-miR-128, np-miR-244, and np-miR-5, which together may be used as indicators for treatment evaluation. The functions of genes targeted by the miRNAs differentially expressed in serofast status patients were further analyzed; these genes were found to be involved in various biological functions, such as T-cell receptor signaling pathways, metabolism, and growth. Our study presents the first systematic landscape of miRNAs in PBMCs from patients with serofast status and proposes specific miRNAs linked with serofast status. Our results provide further evidence that serofast status is closely related to host immune function. Additionally, the miRNA expression profile in PBMCs of patients with serofast status generated by this work offers insight into the complex immune network in humans. We hope our results can provide new insights into the pathogenesis of serofast status.

Keywords Syphilis · Serofast · MicroRNA · Immune system

Abbreviations

| Abbreviation  | Description                                      |
|--------------|--------------------------------------------------|
| PBMC         | Peripheral blood mononuclear cells               |
| miRNA        | MicroRNA                                         |
| TP           | *Treponema pallidum*                             |
| AIDS         | Acquired Immune Deficiency Syndrome              |
| RPR          | Rapid plasma regain                              |
| TPPA         | Particle agglutination assay for antibody to *T. pallidum* |
| FTA-ABS      | Fluorescent treponemal antibody absorption        |
| qPCR         | Real-time fluorescence quantitative PCR           |
| TPM          | Transcripts reads number per million             |
| DEmiRNAs     | Differential expressed miRNAs                    |
| np-miRNAs    | Novel predicted miRNAs                           |

Background
Syphilis is a chronic sexually transmitted disease caused by *Treponema pallidum (T. pallidum)* infection. This bacterium invades various system organs, leading to clinical manifestations such as neurosyphilis, ocular syphilis, and cardiovascular syphilis and seriously endangering human health. After a long course of infection, it can also be asymptomatic and latent; *T. pallidum* may be transmitted to a fetus by vertical transmission [1] and can promote the spread of Acquired Immune Deficiency Syndrome (AIDS) [2–4]. In China, syphilis has become the third leading infectious disease [5],...
and several policies and regulations have been issued to prevent and control this serious public health problem.

Based on its different clinical manifestations, syphilis can be divided into primary syphilis, secondary syphilis, latent syphilis (early and late latent), and tertiary syphilis. According to Chinese national guidelines [6], secondary syphilis is defined as disease characterized by skin rash and possibly lymphadenopathy in a clinically compatible patient combined with laboratory testing results from a rapid plasma reagin (RPR) test, particle agglutination assay for antibody to T. pallidum (TPPA) assay, and/or fluorescent treponemal antibody absorption (FTA-ABS) assay, confirming the presence of T. pallidum in clinical specimens. Some syphilitic patients remain in a serologically positive state even after the recommended therapy; this is referred to as “serofast status”. Many unanswered questions affect the work, life, and family of patients with serofast status, such as whether a serofast patient is contagious, whether female serofast patients should become pregnant, whether a fetus will have congenital syphilis after birth, and whether it will affect related issues like going abroad, marriage examination, and operation. In secondary syphilis patients, serofast status was defined as either no change in the RPR titer or a one-dilution (twofold) decrease or increase in the RPR titer at 6 months after treatment. Those who had a ≥ fourfold decline in their serum RPR titer after the recommended treatment, but showed no seroreversion or any decline in RPR titers for more than one year, were also defined as serofast [7]. The incidence of serofast status amongst treated syphilis patients is variable [7]. When we analyzed the syphilis patients who were treated in our hospital over the past 10 years, the resulting incidence of serofast was 15% [8]. In contrast, Sena et al. [9] reported that the incidence of serofast in patients with early syphilis was 21%, and another recent study showed that up to 73% of patients with serofast early syphilis who were treated with benzathine penicillin continued to maintain their serofast status [10].

In recent years, it has been proposed that cellular immunosuppression occurs after the host is infected with syphilis, resulting in an incomplete elimination of T. pallidum and stimulating the body to continuously produce antibodies, thus leading to the serofast condition. Shetsiruli et al. studied the cellular and humoral immunity of patients with serofast status and found that the overall level of T lymphocytes was decreased, whereas the total levels of B lymphocytes and antigen-reactive cells were elevated [11]. Additionally, the number of CD4+ T cells in HIV-infected patients was found to be significantly reduced after re-infection with syphilis [2, 12, 13]. Carlson et al. summarized the process of clearing T. pallidum from the body, arguing that strong delayed-type hypersensitivity is beneficial for helping the body to clear T. pallidum, whereas a cytotoxic T-cell-mediated immune response and strong humoral immune response will lead to the persistence of infection (serofast) [14]. Our previous work also found that there is a cellular immune imbalance in patients with syphilis [15]. Together, these prior studies suggest that the serofast status is closely related to the immune function of the host. However, how T. pallidum inhibits the immune system and evades immune surveillance to produce a serofast state is still unknown.

MicroRNAs (miRNAs), which are small non-coding RNA molecules of approximately 21 bp in length, can inhibit the mRNA translation of a target gene by binding to the untranslated region of the 3’ end of the mRNA or can regulate the expression of target genes by promoting their degradation. MiRNAs are involved in many important physiological pathways, such as cell proliferation, differentiation, and apoptosis. They not only are important nodes in many pathways but also serve as a new research entry point for revealing the whole picture of these pathways. Lochhead et al. [16] found that miRNAs played an important role in the feedback regulation in arthritis caused by Borrelia burgdorferi infection. Furthermore, miRNAs also perform influential functions during the occurrence and progression of many other infectious diseases [17–22]. Pathogens can use miRNAs for gene expression regulation to facilitate their replication and immune evasion in host cells; for example, miRNA were found to play important roles in the infection processes of Mycobacterium tuberculosis, Helicobacter pylori, and other pathogens [17, 19, 20]. However, studies on whether miRNAs are involved in gene regulation during T. pallidum infection and the potential associated regulatory mechanisms have not been reported.

Our previous study has provided important findings regarding the rapid diagnosis of syphilis [23], load changes of T. pallidum DNA before and after treatment for syphilis [24], and other factors related to the clinical analysis and immunity of serofast status [8]. In this work, we used Illumina sequencing technology to study miRNAs in peripheral blood mononuclear cells (PBMCs) of patients with serofast status, then screened and identified specific miRNAs and investigated their target genes and related regulatory pathways through bioinformatics techniques. We hope our results will provide new insights into the pathogenesis of serofast status.

**Methods**

**Samples**

PBMCs were collected from 28 cases of untreated secondary syphilis, 27 cases of secondary syphilis who remained serofast after treatment (serofast status), and 40 healthy volunteers at the Sexually Transmitted Infection Center of Pecking Union Medical College Hospital from June 2013 to
Peripheral blood (10 ml) was collected using EDTA anticoagulant collecting tube and sent to the laboratory within 20 min. PBMC purification was performed using a Ficoll-Paque gradient centrifugation as previously described [25]. Total RNA was extracted from the PBMCs (1 × 10⁷ cells) using TRIzol reagent (Life Technologies) within 2 hours after blood drawing. 1 × 10⁷ cells in each sample were ensured so as not to lost the miRNAs with low GC content as far as possible [26]. The sampler, pipette tip, EP tube and water involved in the experiment were all processed by DEPC. The entire RNA extraction was performed on ice to minimize the degradation of RNA by RNases. miRNAs were isolated from the total RNA using gel size selection. We then generated miRNA libraries using an Illumina Small RNA Sample Prep Kit (catalog no: FC-102-1009; Illumina Inc., San Diego, CA, USA) in accordance with the manufacturer’s instructions. The Illumina HiSeq 2500 sequencing platform was used for miRNA sequencing.

miRNA identification and analysis of differential expression

miRNAs were identified using the integrated miRNA identification tool miRDeep2 software [27] to obtain known and novel miRNA expression profiles based on the miRNA sequence reads (Additional file: Table S1). The reference genome used to map the sequencing reads is hg38. In miRDeep2, Bowtie [28] was used for read mapping to human genome hg38; reads that mapped perfectly more than five times to the genome were discarded, and one mismatch was allowed to the precursors. RNA structures were predicted by using RNAfold [29]. miRNA sequence reads were also searched against the Rfam database to identify rRNA and snoRNA.

The expressed reads of each miRNA were normalized as the Transcripts Reads Number Per Million (TPM), and the TPM value represents the expression level of miRNA. Lastly, edgeR [30] was used to further identify the differentially expressed (DE) miRNAs (miRNAs with a fold change of > 1 and a p value of < 0.05).

Target gene prediction and function enrichment

Target genes of DEmiRNAs were predicted using miRanda [31] (based on a score of > 140 and free energy of < −20). Target genes of novel predicted miRNAs were further predicted based on their secondary structure hybridizations using the RNAhybrid web tool [32]. GO function and pathway enrichment analyses of these target genes were conducted using DAVID [33].

Real-time quantitative PCR verification

The expression of miRNAs was further confirmed using qPCR assays with specific primers. Total RNA was polyadenylated using ATP and poly(A) polymerase (New England Biolabs, Hitchin, UK) at 37 °C for 1.5 h. The polyadenylated RNA samples were reverse-transcribed into cDNA using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Life Technologies) and a poly(T) adapter to attach a universal tag. Thermo Scientific SYBR Green-based qPCR assays (Life Technologies) were used for determination of miRNA expression levels with a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA). A universal reverse primer was used for these qPCR assays, and the sequences of forward primers were based on the selected miRNA sequence (Additional file: Table S2). For each primer pair, a no-template control to screen for contamination of reagents or false amplification, and a no-RT control with confirm the absence of DNA contamination were included. And each sample was examined in triplicate. The U6 small nuclear RNA gene was used as a control. Thermal cycling conditions involved an initial denaturation at 95 °C for 15 min, followed by 40 amplification cycles (95 °C for 15 s, 56 °C for 20 s, and 68 °C for 20 s). The relative miRNA expression was calculated using the formula $2^{-\Delta Ct} = \frac{\Delta Ct}{{\text{Ct}}(\text{target gene}) - \text{Ct}(\text{reference gene})}$, and $2^{-\Delta Ct}$ reflects the relative expression level of target miRNA.
Statistical analysis was performed using Student’s t test (p value <0.05, *).

**Results**

**Patients**

Twenty-eight patients with secondary syphilis, 27 patients with serofast status, and 40 healthy volunteers from our hospital were enrolled in this study. There were no significant differences in the sex and age distributions among these three patient groups. All patients met the criteria for the diagnosis of syphilis according to Chinese national guidelines [6]. Among the enrolled participants, five subjects from each group were selected for further small RNA transcriptome sequencing (Table 1).

**Overview of global miRNA expression profiles**

Small RNA transcriptomes from the PBMCs of 15 subjects (i.e., five secondary syphilis patients, five serofast status patients, and five healthy volunteers) were sequenced using the Illumina platform. The average number of high-quality sequencing reads in these 15 samples was 26,713,263 with 78% of reads completely mapped to the human genome (Table 1). Among these reads, 73.32% were identified as miRNA transcripts and enriched in 22 bp (Fig. 1). MiRNA expression among five samples in each group showed a high level of consistency (Additional file: Fig. S1).

Using a cutoff of read counts N > 0, we identified a total of 1875, 1791, and 1793 known miRNAs in secondary syphilis, serofast status, and healthy control subjects, respectively. Of these, 1,592 known miRNAs were expressed in samples from all three groups; the remaining 113, 70, and 76 miRNAs were uniquely expressed in the secondary syphilis, serofast status, and healthy control subjects, respectively (Fig. 2a). Interestingly, miR-548b was expressed only in serofast status patients; miR-548b was reported to inhibit the proliferation and invasion of malignant gliomas by targeting metastasis tumor-associated protein-2 [34]. Thus, we thought that miR-548b might have similar functions, inhibiting the proliferation and invasion of *T. pallidum* and leading to a serofast status.

There were 364, 361, and 364 novel predicted miRNAs, of which 6, 3, and 5 were uniquely expressed, discovered in the secondary syphilis, serofast status, and healthy control subjects, respectively (Fig. 2b). Among them, the novel predicted miRNA np-miR-210 (ACAATAGGGTTTACGACC ) was specifically expressed at a high level in serofast status subjects.

To better evaluate the miRNA expression levels in different samples, the read counts were normalized by their TPM values and classified into five levels (Fig. 2c, d). We noticed that the overall expression level was very low (enriched in 0–1), and only ~11% of known miRNAs and less than 0.5% of novel predicted miRNAs had a TPM value of more than 100. The distributions of samples across the five expression levels were similar for all three patient groups.

**DEmiRNA analysis revealed a specific miRNA expression profile and potential markers of serofast status**

An analysis of the DEmiRNAs among secondary syphilis, serofast status, and healthy control subjects was conducted. A total of 146 DEmiRNAs (54 upregulated and 92 downregulated), 111 DEmiRNAs (61 upregulated and 50 downregulated), and 142 DEmiRNAs (88 upregulated and 54 downregulated) was identified between secondary syphilis and healthy control subjects, respectively (Fig. 3). DEmiRNAs with a fold change greater than 2 was also analyzed (Additional file: Fig. S2). The finding that the amount of DEmiRNAs between the serofast status and healthy control subjects was the lowest indicates that the miRNA expression in the serofast status is closer to that in healthy control subjects than that in secondary syphilis patients. In addition, clustering of the identified DEmiRNAs could distinctly classify the 15 samples into three groups (Fig. 4).
Some DEmiRNAs overlapped among the three groups. We identified 15 DEmiRNAs that were upregulated (miR-548bv, miR-889-5p, miR-3135a, miR-144-3p, miR-338-5p, miR-548j-3p, np-miR-194*, miR-196b-3p, miR-548bs, np-miR-210, np-miR-50, miR-3135b, np-miR-284*, miR-208b-3p, and np-miR-211) (Fig. 3e) and five that were downregulated (np-miR-66, miR-7641, miR-3150b-3p, np-miR-3, and miR-6868-3p) (Fig. 3f) in serofast status patients compared with both the healthy control and secondary syphilis subjects. These can be used as potential markers for the assessment of serofast status.
DEmiRNA target gene and function analysis revealed serofast status may be closely related to immunological function

Target genes of the DEmiRNAs were predicted based on the Sanger MicroRNA database. The Unigene database was further used for screening these target genes. A GO analysis of the DEmiRNA target genes revealed that: (1) for secondary syphilis vs. healthy control subjects, the target genes of miRNAs upregulated in secondary syphilis patients were enriched in the ncRNA catabolic process, regulation of double-strand break repair, and positive regulation of cellular carbohydrate, whereas the target genes of miRNAs downregulated in secondary syphilis patients were enriched in the pyruvate metabolic process, lymphocyte co-stimulation, and T-cell co-stimulation (Fig. 5a); (2) for serofast status vs. healthy control subjects, the target genes of miRNAs upregulated in serofast status patients were enriched in histone deacetylation, regulation of receptor activity, and positive regulation of receptor biosynthetic process, whereas the target genes of miRNAs downregulated in serofast status patients were enriched in DNA synthesis involved in DNA repair, the RNA catabolic process, and calcium activated cation channel activity (Fig. 5b); and (3) for serofast status versus secondary syphilis patients, the target genes of miRNAs upregulated in serofast status patients were abundant in positive regulation of alpha–beta T-cell differentiation and calcium-activated potassium channel activity, whereas the target genes of miRNAs downregulated in serofast status patients were enriched in DNA replication, DNA metabolic process, and ion and transmembrane transporter activity (Fig. 5c).

Interestingly, compared with healthy control samples, DEmiRNAs targeted to genes involved in the regulation of...
Fig. 4 Hierarchical clustering of different expressed miRNAs among three groups
of double-strand break repair were upregulated in both the serofast status and secondary syphilis samples. Furthermore, the target genes of miRNAs upregulated in the serofast status samples compared with in the secondary syphilis and healthy control samples were mainly related to receptor biosynthesis or T-cell differentiation, which further implies that the pathogenesis of serofast status is closely related to immunological function.

A KEGG pathway enrichment of the DEmiRNA target genes was also conducted, and the results are shown in Table 2. Compared with healthy control samples, the pathways significantly enriched in patients with serofast status or secondary syphilis included metabolic pathways (e.g., histidine metabolism, fructose and mannose metabolism, tyrosine metabolism, sphingolipid metabolism, and ether lipid metabolism) and a cell growth pathway (Circadian entrainment). Additionally, DEmiRNAs upregulated in serofast status subjects but not in secondary syphilis subjects targeted the neurotrophin signaling pathway and calcium signaling pathway.

DEmiRNAs were further validated by qPCR

To confirm the expression levels of DEmiRNAs, qPCR was conducted. A total of 25 DEmiRNAs (fold-change of >2) in both secondary syphilis and serofast status patients compared with healthy control subjects were selected for further validation (miR-10b, miR-62, miR-122, miR-299, miR-451a, miR548-ar-3p, miR-548av-3p, miR-548az, miR-548j, miR-654, miR-1285f, miR-2478, miR-3135b, miR-3150b, miR-338-5p, miR-3897, miR-4732, np-miR-5, np-miR-73, np-miR-124, np-miR-128, np-miR-163, np-miR-166, np-miR-194, and np-miR-244) (Additional file: Table S3). Due to individual variability, clinical samples always show diverse expression levels. Additionally, the sensitivity of qPCR is not as sensitive as miRNA sequencing. Of these, the qPCR results confirmed 13 miRNAs (miR-62, miR-299, miR-451a, miR-548j, miR-654, miR-3135b, miR-338-5p, np-miR-5, np-miR-73, np-miR-124, np-miR-128, np-miR-163, np-miR-166, np-miR-194, and np-miR-244) (Additional file: Table S4).

These 13 DEmiRNAs confirmed by qPCR were further verified in additional samples (23 secondary syphilis, 22 serofast status, and 40 healthy control subjects); these results validated five miRNAs (miR-338-5p, np-miR-5, np-miR-128, np-miR-163, and np-miR-244). Among these five miRNAs, np-miR-128 and np-miR-244 were downregulated in the PBMCs of secondary syphilis and serofast status patients; np-miR-163 was downregulated and np-miR-5 was upregulated in PBMCs from secondary syphilis patients; and the known miR-338-5p was upregulated in PBMCs from serofast status patients (Additional file: Table S4 and Fig. S3).

miR-338-5p, upregulated in serofast status patients, might be a potential biomarker of serofast status

In the present study, we also predicted the miR-338-5p target genes and conducted a GO function enrichment of these miR-338-5p target genes (Fig. 6). We found that the functions were mainly involved ion or protein transport, and many of these biological processes required RANBP17, XPO1, and XPO6. In microbial infection, XPO1 mediates the export by different viruses (e.g., HIV-1, HTLV-1, and influenza A) of unspliced or incompletely spliced RNAs out of the nucleus [35, 36]. RANBP17 and XPO6 are also involved in protein import to the nucleus or export from the nucleus. Therefore, we speculate that in the PBMCs of serofast status patients, miR-338-5p might play an important role in regulating the infection process of T. pallidum by targeting RANBP17, XPO1, or XPO6, thus reducing the deleterious effects of T. pallidum.

Discussion

To date, no breakthroughs had been made on the pathogenesis of serofast status. Clinically, even without treatment, the RPR titer in some patients with syphilis will gradually decline to levels below detection limits, indicating that the immune system condition of the host strongly affects the prognosis of syphilis. Our previous studies suggested that immunologic imbalance and immunosuppression may appear after T. pallidum infection, leading to the incomplete elimination of T. pallidum. However, the results of IgM antibody detection revealed that serofast status patients have minimal infectivity, indicating that the status of immunologic imbalance might be an important cause of the serofast status. In this case, the immunologic imbalance is characterized by a secretion disorder of T-cell subsets, NK cells, and cytokines along with an imbalance of CD4+CD8+ T cells and Th1/Th2 cytokines, resulting in the reduction of delayed-type hypersensitivity to T. pallidum antigen and further influencing the prognosis of syphilis patients [37]. Most prior studies focused on the immunological features of serofast status patients in the hope that understanding the immune response to infection with T. pallidum would provide insight into the pathogenesis of serofast status.

MiRNAs, which are small RNA (~21 bp long) that post-transcriptionally regulate gene expression, are involved in many important physiological and pathological pathways, including inflammation, tissue repair, apoptosis, and immune responses [38, 39]. During microbial infection, miRNAs up/downregulate the host innate immune response through the Toll-like receptor (TLR) pathway and the associated cytokines production [40].
The performance of miRNAs is complex and bidirectional. In some cases, miRNAs from pathogens participate in the regulation of self-replication and survival in the host. However, host miRNAs are involved in both innate and acquired immune response processes that can inhibit the proliferation of pathogens [39, 42, 43]. Despite their known involvement with other infectious diseases, studies on miRNA related to *T. pallidum* infection, and especially the serofast status, are lacking. The miRNA expression profile of PBMCs from serofast status patients can provide insight into the complex immune network. Immune system biomarkers for different infection statuses can also be detected and then applied to the diagnosis, treatment, and prognosis of this condition [44–47].

Here, small RNA transcriptomes of PBMCs from serofast status and secondary syphilis patients were studied by using deep-sequencing and qPCR. We clarified the differences in molecular expression and associated pathways between these two types of patients. In terms of RNA extraction, we used TRIzol reagent. Previous studies have shown that Trizol extraction specifically enriches for some types of miRNAs and does not adequately extract miRNAs with low GC content [26]. To reduce the influence of Trizol, $1 \times 10^7$ PBMCs in each sample were ensured. We further analyzed the GC content of detected miRNAs, the result showed that the GC content of miRNAs ranged from 12.5 to 96% (Additional file: Fig. S4), which indicated that the results were not affected by Trizol. In addition, miRNA sequencing is becoming very mature and stable with the development of sequencing technology. Therefore, biological duplication is more biologically meaningful than technical replicates, and has become one aspect that considered in experimental design [48, 49]. In this study, PBMCs of five individuals were selected from each of the three groups (syphilis, serofast status, and healthy

Table 2  KEGG pathway enrichment of target genes of different expressed miRNAs

| Function ------------------------------------------------- | KEGG pathway | P value |
|--------------------------------------------------------|--------------|---------|
| Secondary syphilis versus healthy control              |              |         |
| Up in secondary syphilis                               | Circadian entrainment | hsa04713 | 0.010  |
|                                                      | Glioma       | hsa05214 | 0.036  |
| Down in secondary syphilis                             | Histidine metabolism | hsa00340 | 0.010  |
|                                                      | Fructose and mannose metabolism | hsa00051 | 0.030  |
|                                                      | Hippo signaling pathway | hsa04390 | 0.042  |
|                                                      | T cell receptor signaling pathway | hsa04660 | 0.044  |
| Serofast status versus healthy control                 |              |         |
| Up in serofast status                                  | Transcriptional misregulation in cancer | hsa05202 | 0.020  |
|                                                      | HTLV-1 infection | hsa05166 | 0.025  |
|                                                      | Circadian entrainment | hsa04713 | 0.035  |
|                                                      | Fat digestion and absorption | hsa04975 | 0.039  |
|                                                      | Viral myocarditis | hsa05416 | 0.045  |
| Down in serofast status                                | Vascular smooth muscle contraction | hsa04270 | 0.018  |
|                                                      | Tyrosine metabolism | hsa00350 | 0.029  |
|                                                      | Fat digestion and absorption | hsa04975 | 0.033  |
|                                                      | Sphingolipid metabolism | hsa00600 | 0.043  |
|                                                      | Ether lipid metabolism | hsa00565 | 0.045  |
| Serofast status versus secondary syphilis              |              |         |
| Up in serofast status                                  | Neurotrophin signaling pathway | hsa04722 | 0.018  |
|                                                      | Calcium signaling pathway | hsa04020 | 0.031  |
|                                                      | Nicotine addiction | hsa05033 | 0.036  |
|                                                      | Alzheimer’s disease | hsa05010 | 0.049  |
| Down in serofast status                                | Phosphatidylinositol signaling system | hsa04070 | 0.031  |
|                                                      | Histidine metabolism | hsa00340 | 0.039  |
|                                                      | Insulin signaling pathway | hsa04910 | 0.042  |
|                                                      | Adipocytokine signaling pathway | hsa04920 | 0.045  |
volunteers) for RNA sequencing. We also did a consistency analysis, and the results showed a high consistency among 5 samples in each group (Additional file: Fig. S1). High consistency ensured the reliability of subsequent analysis. In regard to the criteria of different expressed miRNAs, a fold change of > 1.2 together with a p value of < 0.05 was selected. Actually, the selection of threshold of fold change is variable, some papers use 1.2 [50, 51], some papers use 1.5 [48, 52] and some use 2 [49, 53]. In our study, when we set the threshold p value < 0.05, we found that the smallest fold change is 1.32. Therefore, we chose a relatively low threshold so as not to miss important results. Still, the different expressed miRNAs with fold change greater than 2 were the main object of attention. Through an initial bioinformatics analysis and further confirmation with additional samples, we discovered that miR-338-5p was upregulated in serofast status patients compared with secondary syphilis and healthy control subjects. This miRNA was reported to be involved in many important biological processes: miR-338-5p can regulate the viability, proliferation, apoptosis, and migration of rheumatoid arthritis fibroblast-like synoviocytes by targeting NFAT5; miR-338-5p promotes glioma cell invasion by regulating TSHZ3 and MMP2; miR-338-5p modulates B-cell biological functions by targeting NF-κB1; miR-338-5p is closely correlated with the procedure of renal allograft antibody-mediated rejection [54–57]; and miR-338-5p can also indirectly regulate the BAFF signaling pathway by targeting immune cell cytokines such as TRAF3, participating in the immune response after renal transplantation and effecting the long-term survival of a transplanted kidney [58]. These results indicate that miR-338-5p might be related to immunologic functions.

Several novel predicted miRNAs were found to be differentially expressed in serofast status patients; for example, np-miR-128 and np-miR-24 were downregulated in serofast status patients. We analyzed the function and pathway enrichment for the target genes of these DEmiRNAs, and the results indicate that the highly enriched functions include metabolism (e.g., histidine metabolism, fructose and mannose metabolism, tyrosine metabolism, sphingolipid metabolism, and other lipid metabolism), growth (e.g., cell proliferation and cell cycle), and differentiation and apoptosis (e.g., cell apoptosis, anti-apoptosis, induced apoptosis, and

**Fig. 6** Expression levels and significant GO terms of target genes of miR-338-5p.

a Expression level (qPCR) of miR-338-5p in three groups (p < 0.05, *).

b Significant GO terms of target genes of miR-338-5p.
inhibition of apoptosis). Therefore, research on miRNAs and their target genes may be able to provide insights into the pathogenesis of secondary syphilis, especially the serofast status. Many miRNAs have been found to play important roles in immune system self-stabilization and anti-infection immune regulation, such as Let-7, miR-146, miR-155, miR-182, and miR-185 [59–62]. Our findings suggest that miRNAs might also play important roles in serofast status patients, including the novel predicted miRNAs np-miR-128 and np-miR-24.

Conclusions

Here, we conducted a systematic investigation of the miRNA expression profile in PBMCs from patients with serofast status and compared it with the miRNA expression profiles in PBMCs from secondary syphilis and healthy control subjects. Our results display a specific miRNA expression profile in serofast status patients. Both known and novel predicted miRNAs were identified and verified, such as miR-338-5p, np-miR-163, np-miR-128, np-miR-244, and np-miR-5. The target genes of the DEmiRNAs in serofast status patients were found to be involved in various biological functions, such as T-cell receptor signaling pathway, metabolism, and growth. Our study presents the first systematic landscape of miRNAs in PBMCs from patients with serofast status. Furthermore, our results possibly indicate that serofast status is closely related to the host immune function, yet more work is needed. The miRNA expression profile of PBMCs from serofast status patients that we present here provides insight into the complex human immune network. We hope our results will improve insight into the pathogenesis of serofast status.

Acknowledgements We thank Rongxin Ren, M.D., in the Department of Plastic and Aesthetic Surgery, Beijing Hospital, Beijing, China.

Author contributions LJ conceived and supervised the project. JX, LJ, and ZH designed and implemented the methods, and conducted the experiments. WZ and LX contributed to data acquisition. JX, and LJ wrote the manuscript. All authors approved the manuscript.

Funding Noe.

Data availability The datasets used and/or analyzed during the current study are available from the Additional files.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval All participants provided informed written consent for all study procedures and for the use of their data for scientific evaluation and publication in a blinded form. This study was conducted in accordance with the Declaration of Helsinki, and was approved by the ethics committee of Peking Union Medical College Hospital (reference number S-K102).

References

1. Mabey D, Peeling RW (2011) Syphilis, still a major cause of infant mortality. Lancet Infect Dis 11(9):654–655
2. Jarzabowski W, Caumes E, Dupin N et al (2012) Effect of early syphilis infection on plasma viral load and CD4 cell count in human immunodeficiency virus-infected men: results from the FHDH-ANRS CO4 Cohort. Arch Intern Med 172(16):1237–1243
3. Taylor MM, Li WW, Skinner J et al (2014) Viral loads among young HIV-infected men with early syphilis. J Int Assoc Provid AIDS Care 13(6):501–505
4. Niedermeier A, Kovnerystyy O, Braun-Falco M (2010) Syphilis in the context of HIV-infection: a complex disease. Dtsch Med Wochenschr 135(28–29):1423–1426
5. Chen XS, Yin YP, Wang QQ et al (2013) Historical perspective of syphilis in the past 60 years in China: eliminated, forgotten, on the return. Chin Med J 126(14):2774–2779
6. SBTS/MOH (1996) National standard of the People’s Republic of China: diagnostic criteria and management of syphilis (GB 15974-1995). Standards Press of China, Beijing
7. Sena AC, Zhang XH, Li T et al (2015) A systematic review of syphilis serological treatment outcomes in HIV-infected and HIV-uninfected persons: rethinking the significance of serological non-responsiveness and the serofast state after therapy. BMC Infect Dis 15:479
8. Li J, Wang LN, Zheng HY (2013) Predictors of serological cure and serofast state after treatment in HIV-negative patients with early syphilis in China. Sex Transm Infect 89(1):69–69
9. Sena AC, Wolff M, Martin DH et al (2011) Predictors of serological cure and serofast state after treatment in HIV-negative persons with early syphilis. Clin Infect Dis 53(11):1092–1099
10. Sena AC, Wolff M, Behets F et al (2013) Response to therapy following retreatment of serofast early syphilis patients with benzathine penicillin. Clin Infect Dis 56(3):420–422
11. Shetsiruli LT, Gogebashvili NV, Toniai KS (1989) Immunologic aspects of the pathogenesis of latent and seroresistant forms of syphilis. Vestn Dermatol Venerol 5:55–58
12. Kofied K, Gerstoft J, Mathiesen LR et al (2006) Syphilis and human immunodeficiency virus (HIV)-1 infection: influence on CD4 T-cell count, HIV-1 viral load, and treatment response (vol 33, pg 413, 2006). Sex Transm Dis 33(5):336–336
13. Buchacz K, Patel P, Taylor M et al (2004) Syphilis increases HIV viral load and decreases CD4 cell counts in HIV-infected patients with new syphilis infections. AIDS 18(15):2075–2079
14. Carlson JA, Dahiri G, Gribier B et al (2011) The immunopathobiology of syphilis: the manifestations and course of syphilis are determined by the level of delayed-type hypersensitivity. Am J Dermatopathol 33(5):433–460
15. Li J, Wang LN, Zuo YG et al (2009) Clinical analysis and study of immunological function in syphilis patients with seroresistance. Zhonghua Yi Xue Za Zhi 89(12):813–816
16. Lochhead RB, Ma Y, Zachary JF et al (2014) MicroRNA-146a provides feedback regulation of lyme arthritis but not carditis during infection with Borrelia burgdorferi. PLoS Pathog 10(6):e1004212
17. Chang H, Kim N, Park JH et al (2015) Different microRNA expression levels in gastric cancer depending on Helicobacter pylori infection. Gut Liver 9(2):188–196
...
58. Xu H, He X, Xu R et al (2013) miR-338-5p indirectly regulate BAFF signal by targeting TRAF3 during renal allograft antibody-mediated rejection (P2200). J Immunol 190(1 Supplement):69.38
59. Akira S, Maeda K (2017) Introduction: dynamics of RNA regulation in the immune system special issue. Int Immunol 29(4):145–147
60. Ansel KM (2013) RNA regulation of the immune system. Immunol Rev 253:5–11

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Affiliations

Xinmiao Jia1 · Zhongshuai Wang2 · Xiaoke Liu2 · Heyi Zheng2 · Jun Li2

Xinmiao Jia  
jiaxinmiaohappy@126.com
Zhongshuai Wang  
iamtheoneee@163.com
Xiaoke Liu  
565031125@qq.com
Heyi Zheng  
zhenghy62@hotmail.com

1 Medical Research Center, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, No.1 Shuaifuyuan Wangfujing Dongcheng District, Beijing 100730, China
2 Department of Dermatology and Venereology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China