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

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Abstract

Background 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. **Methods** 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. **Results** 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 that merit further investigation as potential biomarkers. **Conclusion** 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 inspire the development of new treatment options for patients with serofast status.

Background

Syphilis is a chronic sexually transmitted disease caused by *Treponema pallidum* infection. This bacteria 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 viral infectious disease after viral hepatitis and tuberculosis [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, 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 can be 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, at 6 months following treatment, serofast was defined as either no change in the RPR titer or a one-dilution (two-fold) decrease or increase in the titer following treatment. Those who had a ≥ 4 -fold 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. The incidence of serofast is inconsistent between reports [6]. When we analyzed the syphilis patients who were treated in our hospital over the past 10 years, the resulting incidence of serofast was 15% [7]. In contrast, Sena et al [8] 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 [9].

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 [10]. Additionally, the number of CD4⁺ T cells in HIV-infected patients was found to be significantly reduced after re-infection with syphilis [2, 11, 12]. Carlson et al summarized the process of clearing *T. pallidum* from the body, arguing that strong delayed-type hypersensitivity is beneficial from 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) [13]. Our previous work also found that there is a cellular immune imbalance in patients with syphilis [14]. 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 [15] 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 [16-21]. 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 [16, 18,

19]. However, studies on whether miRNAs are involved in regulation during *T. pallidum* infection and the associated regulatory mechanisms have not been reported. Previous work has provided a important findings regarding the rapid diagnosis of syphilis, detection of anti-*T. pallidum* IgM antibody through western blot, load changes of *T. pallidum* DNA before and after treatment for syphilis, and other factors related to the clinical analysis and immunity of serofast status. Based on the previous studies, the present work 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 syphilis and new treatment options for 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 June 2014. According to Chinese national guidelines [22], secondary syphilis is defined as the presentation of disease in a clinically compatible patient characterized by skin rash, often accompanied by lymphadenopathy, and laboratory test results from an RPR, TPPA, and/or FTA-ABS test confirming the presence of *T. pallidum* in clinical specimens. In the secondary syphilis patients, serofast was defined as either no change in the RPR titer or a one-dilution (two-fold) decrease or increase in the titer following treatment (benzathine penicillin G, 2.4 million units intramuscular weekly) for 6 months. Patients who had a ≥ 4 -fold 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. Additionally, pregnant or breast-feeding women and patients who were HIV antibody-positive or had neurosyphilis, tuberculosis, an autoimmune disease, cancer, chronic liver disease, kidney disease, and another systemic disease were excluded from enrolling in our study. Five individuals were selected from each of the three groups (total of 15 subjects); their PBMCs were send for further RNA sequencing, and their other samples were used for further miRNA real-time fluorescence quantitative PCR (qPCR) validation.

PBMC collection and miRNA sequencing

EDTA anticoagulant tubes were used for collecting peripheral blood samples (10 ml). PBMC purification was performed using a Ficoll-Paque gradient centrifugation as previously described [23]. Total RNA was extracted from the PBMCs using TRIzol reagent (Life Technologies). 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 [24] 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 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 [25]. 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 [26] 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 DE miRNAs were predicted using miRanda [27] (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 [28]. GO function and pathway enrichment analyses of these target genes were conducted using DAVID [29].

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). 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 $\Delta Ct = [Ct(\text{target gene}) - Ct(\text{reference gene})]$, and $2^{-\Delta Ct}$ reflects the relative expression level of target miRNA.

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 [22]. Pregnant or breast-feeding women and patients who were HIV antibody-positive or had neurosyphilis, tuberculosis, an autoimmune disease, cancer, chronic liver disease, kidney disease, or another systemic disease were excluded from enrollment. 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).

Using a cutoff of read counts $N > 0$, we identified a total of 1,875, 1,791, and 1,793 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 [30]. Thus, we thought that miR-548b might have similar functions, inhibiting the proliferation and invasion of *T. pallidum* and leading to a low RPR titer.

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. 2cd). 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, serofast status and healthy control subjects, and serofast status and secondary syphilis subjects, respectively (Fig. 3). 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 to 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 vs. 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 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, miR-548-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. 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, 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. S1).

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 in 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 [31, 32]. 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 [33]. 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 [34, 35]. During microbial infection, miRNAs up/downregulate the host innate immune response through the Toll-like receptor (TLR) pathway and the associated cytokines production [36, 37]. 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 [35, 38, 39]. 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 [40-43].

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. 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- κ B1; miR-338-5p is closely correlated with the procedure of renal allograft antibody-mediated rejection [44-47]; 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. 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 ether lipid metabolism), growth (e.g., cell proliferation and cell cycle), and differentiation and apoptosis (e.g., cell apoptosis, anti-apoptosis, induced apoptosis, and 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 [48-51]. 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 health 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; these may be used clinically as an indicator for evaluating treatment success. 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 and proposes specific miRNAs as potential biomarkers for serofast status that should be experimentally validated in future work. Furthermore, our results indicate that serofast status is closely related to the host immune function. 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 and inspire new treatment options for serofast status.

Abbreviations

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 *Treponema 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.

Declarations

Ethics approval and consent to participate

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).

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

L.J. conceived and supervised the project. J.X., L.J., and Z.H. designed and implemented the methods, and conducted the experiments. W.Z. and L.X. contributed to data acquisition. J.X., and L.J. wrote the manuscript. All authors approved the manuscript.

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Tables

Table 1. Clinical information of syphilitic patients with Peripheral blood mononuclear cell sequenced

Diagnosis	Sex	Age	Course of disease	RPR $\emptyset$ Titer
Secondary syphilis	Male	20-24	2 weeks	$\geq 1:32$
Secondary syphilis	Famale	30-34	2 months	$\geq 1:32$
Secondary syphilis	Male	25-39	1 month	$\geq 1:32$
Secondary syphilis	Famale	30-34	2 weeks	$\geq 1:32$
Secondary syphilis	Male	45-50	1 month	$\geq 1:32$
Serofast status	Male	50-54	3 years*	1:8
Serofast status	Famale	20-24	1.5 years*	1:16
Serofast status	Famale	30-34	1.5 years*	1:16
Serofast status	Famale	20-24	2 years*	1:4
Serofast status	Famale	35-39	2 years*	1:8
Health control	Male	20-24	NA	NA
Health control	Famale	45-50	NA	NA
Health control	Male	45-50	NA	NA
Health control	Male	30-34	NA	NA
Health control	Female	30-34	NA	NA

$\emptyset$: Rapid plasma regain

*: Secondary syphilis patients that had a ≥ 4 -fold decline in their serum RPR titer after the recommended treatment, but showed no seroreversion or any decline in RPR titers for more than 1.5-3 years.

NA: Not applicable

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

		Function	KEGG pathway	p-Value
Secondary syphilis vs. 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
Serofast status vs. Healthy control	Up in serofast status	T cell receptor signaling pathway	hsa04660	0.044
		Transcriptional misregulation in cancer	hsa05202	0.020
		HTLV-I infection	hsa05166	0.025
		Circadian entrainment	hsa04713	0.035
		Fat digestion and absorption	hsa04975	0.039
	Down in serofast status	Viral myocarditis	hsa05416	0.045
		Vascular smooth muscle contraction	hsa04270	0.018
		Tyrosine metabolism	hsa00350	0.029
		Fat digestion and absorption	hsa04975	0.033
		Sphingolipid metabolism	hsa00600	0.043
Serofast status vs. Secondary syphilis	Up in serofast status	Ether lipid metabolism	hsa00565	0.045
		Neurotrophin signaling pathway	hsa04722	0.018
		Calcium signaling pathway	hsa04020	0.031
		Nicotine addiction	hsa05033	0.036
	Down in serofast status	Alzheimer's disease	hsa05010	0.049
		Phosphatidylinositol signaling system	hsa04070	0.031

Figures

Figure 1

Distribution of miRNA sequencing reads. a. Distribution ration of miRNA sequencing reads. b. Length distribution of miRNA sequencing reads.

Figure 2

Global miRNA expression profiles. a. Overlaps of expressed (TPM > 0) known miRNAs. b. Overlaps of expressed (TPM > 0) novel predicted miRNAs. c. Distribution of known miRNA expression levels. X axis was divided into six parts by five expression levels and the sixth total expression counts. Y axis indicated TPM of miRNAs according to expression levels. d. Distribution of novel predicted miRNA expression levels. X axis was divided into six parts by five expression levels and the sixth total expression counts. Y axis indicated TPM of miRNAs according to expression levels.

Figure 3

Different expressed miRNAs. a. Different expressed miRNAs between secondary syphilis and health control. Up-expressed miRNAs in secondary syphilis were shown as red dots, and down-expressed ones were marked as green dots. b. Different expressed miRNAs between serofast status and health control. Up-expressed miRNAs in serofast status were shown as red dots, and down-expressed ones were marked as green dots. c. Different expressed miRNAs between serofast status and secondary syphilis. Up-expressed miRNAs in serofast status were shown as red dots, and down-expressed ones were marked as

green dots. d. Number of different expressed miRNAs corresponding to figure 3a, figure 3b and figure 3c. “A” represents different expressed miRNAs between secondary syphilis and health control in figure 3a. “B” represents different expressed miRNAs between serofast status and health control in figure 3b. “C” represents different expressed miRNAs between serofast status and secondary syphilis in figure 3c. “Red” and “green” have the same meanings with that in figure 3a, figure 3b and figure 3c, respectively. e. Overlaps of up-expressed miRNAs. f. Overlaps of down-expressed miRNAs.

Figure 4

Hierarchical clustering of different expressed miRNAs among three groups.

Figure 5

Top 10 GO function enriched terms of target genes of different expressed miRNAs. a. secondary syphilis vs. health control. b. serofast status vs. health control. c. serofast status vs. secondary syphilis. Black bars represent the target genes of up-regulated miRNAs in the former group, and white bars means the target genes of down-regulated miRNAs in the former group.

Figure 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.

Supplementary Files

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