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Concise report

Pneumocystis pneumonia and rheumatic disease: diagnostic potential of circulating microbial cell-free DNA sequencing

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Abstract

Objectives. The aim of this study was to explore the clinical utility of circulating microbial cell-free DNA (cfDNA) sequencing as a non-invasive approach for diagnosis of *Pneumocystis jirovecii* pneumonia (PJP) in immunocompromised patients with rheumatic disease (RD).

Methods. The study included 72 RD patients with suspected lung infections admitted to Renji hospital. Eighteen individuals were diagnosed with PJP, and 54 patients without PJP were enrolled as the control group. All patients had undergone pulmonary CT scans, and blood and respiratory tract specimens had been subjected to metagenomic next-generation sequencing (mNGS) and conventional microbiological tests. The clinical and laboratory parameters were collected, and the efficacy of circulating microbial cfDNA of *P. jirovecii* was evaluated.

Results. Of the 18 patients with PJP, the average age was 53.0 years, and the median time between RD diagnosis and PJP presentation was 126.0 days (interquartile range 84.0–176.3 days). Low circulating $CD4^+$ cell counts and a lack of PJP prophylaxis were observed in the patients. Metagenomic NGS of circulating microbial cfDNA was performed in 69 patients, including 15 cases with PJP and 54 controls. Twelve (80%) of 15 analysed blood samples contained *P. jirovecii* sequences in the PJP group, with *P. jirovecii* not detected among controls. There was a significant difference between PJP and non-PJP groups ($P < 0.001$), with a sensitivity of 83.3% and specificity of 100% when using plasma cfDNA sequencing. Higher β-D-glucan levels were found in patients with positive results for *P. jirovecii* in plasma cfDNA sequencing.

Conclusion. Metagenomic NGS of circulating microbial cfDNA is a potential tool for diagnosis of PJP in RD patients.

Key words: circulating microbial cell-free DNA; next-generation sequencing; Pneumocystis jirovecii pneumonia; rheumatic disease; anti-MDA5 antibody-positive dermatomyositis

CLINICAL SCIENCE

Key messages

- Low CD4 cell counts without *Pneumocystis jirovecii* pneumonia prophylaxis are related to *P. jirovecii* pneumonia in rheumatic patients.
- Analysis of circulating cell-free DNA is a non-invasive approach for identifying pathogens in respiratory tract infections.
- Metagenomic next-generation sequencing of microbial cell-free DNA could be applied for *P. jirovecii* pneumonia detection.

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Introduction

Patients with autoimmune and autoinflammatory rheumatic disorders are at an increased risk of opportunistic infections, including *Pneumocystis jirovecii* pneumonia (PJP), owing to a weakened immune response as a result of treatment with glucocorticoids and immunosuppressants [1]. As one of the leading types of severe pneumonia, PJP is associated with a high mortality rate in immunocompromised patients with rheumatic disease (RD) [2]. Early diagnosis of PJP is the key to improving clinical prognosis. PJP is diagnosed based on integrated clinical, radiographic and microbiological criteria. However, identification of *P. jirovecii* infection remains challenging because of limitations in its cultivation *in vitro* and diagnostic microbiological methods.

Over the past few decades, the most commonly used laboratory diagnostic methods for PJP have been tinctorial or immunofluorescence staining assays of bronchoalveolar lavage fluid samples or induced sputum [3, 4]; however, microscopic detection has low sensitivity and specificity [5]. Alternative approaches based on nucleic acid amplification of respiratory specimens are more sensitive than immunofluorescence, although the results might vary across clinical specimens because of a density gradient load of *P. jirovecii* from the upper respiratory tract to the alveoli [3]. Moreover, these techniques require bronchoscopy, an invasive procedure that requires specially trained personnel, which might be unacceptable or unfeasible in some cases.

Plasma cell-free DNA (cfDNA) comprises small fragments of circulating DNA in the cell-free compartment of peripheral blood. Previous studies have demonstrated that fragments of genomic DNA from pathogens in various organs of the body can be detected in the peripheral blood of patients [6]. As a non-invasive approach, microbial cfDNA sequencing based on metagenomic next-generation sequencing (mNGS) has shown remarkable potential for identification of a wide range of pathogens, including opportunistic infections [7]. However, only a few studies have addressed the application of plasma microbial cfDNA in the diagnosis of PJP. In the present study, we aimed to describe the clinical features of PJP and evaluate the application of cfDNA sequencing to the diagnosis of PJP in individuals with RD.

Methods

Study design and patients

From January 2019 to January 2021, 72 hospitalized rheumatic patients with suspected lung infections were recruited at the Department of Rheumatology, Renji Hospital, Shanghai Jiao Tong University School of Medicine. All the enrolled patients met one or more of the classification criteria for RD as follows: SLE, RA, DM, ANCA-associated vasculitis, adult-onset Still's disease and undifferentiated CTD. All patients had undergone pulmonary CT scans and mNGS for pathogen identification upon admission. Infections were suspected in patients with fever, respiratory symptoms or new lung lesions. Pathogens were identified with a panel of conventional microbiological tests and mNGS. *Pneumocystis jirovecii* sequences were identified in plasma cfDNA or respiratory samples using mNGS in patients. Infections were diagnosed by two independent clinicians, based on the clinical manifestations, laboratory tests, radiological demonstration and microbiological tests [8]. Demographic information, clinical parameters and treatment regimen data of patients were collected and analysed.

This study was approved by the Ethics Committee of Renji Hospital, Shanghai Jiao Tong University School of medicine (No. 2017-Clinical-Prs-201). All participants gave written informed consent.

Specimen collection, processing and detection

Specimens were collected from patients according to the standard procedure. Briefly, 3 ml of blood, bronchoalveolar lavage fluid or sputum was collected in sterile, DNase-free tubes. Plasma was separated by centrifuging blood at 1,600g at 4°C for 10 min within 8 h of collection. Nucleic acid was extracted using TIANamp Micro DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. The extracted DNA specimens were used for the construction of DNA libraries [9]. Quality-qualified libraries were pooled, and DNA Nanoball (DNB) was generated. DNA was sequenced using the MGISEQ-2000 platform (MGI-tech, Shenzhen, China) [3]. High-quality sequencing data were generated by removing low-quality reads, followed by computational subtraction of human host sequences mapped to the human reference genome (hg19) using Burrows–Wheeler alignment [10]. The remaining data, after removal of low-complexity reads, were classified via simultaneous alignment with the Pathogens Metagenomics Database. The reference databases for classification were downloaded from NCBI. RefSeq contains 6350 bacterial genomes or scaffolds, 1064 fungi related to human infection, 4945 whole-genome sequences of viral taxa and 234 parasites associated with human diseases. The number of *P. jirovecii* was defined as the number of unique reads of standardized species.

Statistical analysis

Statistical analyses were performed using SPSS (IBM SPSS Statistics v.22). Continuous variables were expressed as the mean (s.d.) for normal distributions and as the median and interquartile range (IQR) for non-normal distributions. Categorical variables were expressed as absolute frequencies and percentages. The differences were analysed via the Mann–Whitney *U*-test, χ^2 or Fisher's exact tests based on the type of data and distribution. A two-sided *P*-value <0.05 was considered statistically significant.

Results

All the RD patients were categorized into two groups: PJP and non-PJP. Eighteen patients were diagnosed

Table 1 The demographic and clinical characteristics of patients with *Pneumocystis jirovecii* pneumonia and rheumatic disease

Characteristics	PJP($n = 18$)	Non-PJP($n = 54$)	*P*-value	Reference range
Age, years	53.0 ± 16.2	53.5(36.8-63.3)	0.545	
Female sex, *n* (%)	10 (55.6)	42 (77.8)	0.068	
All-cause mortality, *n* (%)	9 (50.0)	10 (18.5)	0.009	
Disease duration, days	126.0 (84.0–176.3)	90 (43.75–202.5)	0.349	
Prednisone exposure (dose ≥ 20 mg*1M)	18 (100.0)	40 (74.1)	0.016	
Exposure to csDMARDs, *n* (%)	18 (100.0)	52 (96.3)	0.408	
Exposure to bDMARDs, *n* (%)	2 (11.1)	10 (18.5)	0.465	
TMP-SMX prophylaxis, *n* (%)	0 (0)	5 (9.3)	0.181	
Absolute CD4 cell count, cells/μl	162.0 (78.4–298.8)	273.5 (135.2–522.47)	0.006	447–1030
Lactate dehydrogenase, U/l	505.0 (373.0–710.3)	319.5 (208.75–460)	0.004	120–250
β-D-glucan, pg/ml	199.7 (88.0–352.9)	10.0 (10.0–54.2)	0.000	<60
Positive β-D-glucan, *n* (%)	14 (77.8)	12 (22.2)	0.000	
Co-morbidities, *n* (%)				
Hypertension	4 (22.2)	12 (22.2)	1.000	
Diabetes	6 (33.3)	12 (22.2)	0.346	
Cancer	1 (5.6)	4 (7.4)	0.789	
Interstitial lung disease	13 (72.2)	24 (44.4)	0.041	
Symptoms, *n* (%)				
Fever	15 (83.3)	47 (87.0)	0.694	
Cough	13 (72.2)	38 (70.4)	0.881	
Dyspnoea	16 (88.9)	39 (72.2)	0.149	
Treatment, *n* (%)				
TMP-SMX	18 (100.0)			
Caspofungin	17 (94.4)			

Continuous variables are expressed as the mean (S.D.) for normal distributions, and as the median and interquartile range (IQR) for non-normal distributions. Categorical variables are expressed as absolute frequencies and percentages. bDMARDs: biological DMARDs; csDMARDs: conventional synthetic DMARDs; PJP: *Pneumocystis jirovecii* pneumonia; RD: rheumatic disease; TMP-SMX: trimethoprim–sulfamethoxazole.

with confirmed PJP. The non-PJP group comprised 39 patients with non-PJ infectious pneumonia and 15 patients with pulmonary lesions owing to primary RD. The demographic and clinical characteristics of patients are summarized in Table 1. The average age in the PJP group was 53.0 years, and 55.6% of the patients were female. The median time between RD diagnosis and PJP presentation was 126.0 days (IQR 84.0–176.3 days). All patients were administered ≥20 mg of prednisone daily and were prescribed conventional synthetic DMARDs (csDMARDs) before PJP. Two of the patients were administered additional biologic DMARDs (bDMARDs), including etanercept or tocilizumab. Dyspnoea and fever were the most common symptoms in patients with PJP and RD (88.9% and 83.3%, respectively). Fourteen patients showed positive β-D-glucan testing. Circulating $CD4^+$ cell counts <200 cells/μl and a lack of PJP prophylaxis were identified as the risk factors for PJP infection. All patients were administered oral trimethoprim–sulfamethoxazole therapy, and 94.4% received it in combination with i.v. caspofungin, which is currently used to treat PJP. All-cause mortality was 50% in patients with PJP.

Of the 18 patients, 8 (44.44%) exhibited DM, 3 (16.67%) had SLE or adult-onset Still's disease, 2 (11.10%) had ANCA-associated vasculitis, and 1 (5.56%) had RA or undifferentiated CTD. Metagenomic NGS was conducted on 22 samples, and plasma microbial cfDNA sequencing was performed with 15 peripheral blood samples. Twelve (80%) of the 15 blood samples contained *P. jirovecii* sequences. The specific reads of *P. jirovecii* ranged from 1 to 297, as determined using circulating cfDNA sequencing (Table 2). Proportions of *P. jirovecii* specific reads in fungi were 100% in 11 patients. *Candida albicans* reads were detected in the plasma cfDNA from case 8. In contrast, metagenomic NGS of circulating microbial cfDNA did not find *P. jirovecii* sequences in 54 patients of the control group. There was a significant difference between PJP and non-PJP groups ($P < 0.001$), with a sensitivity of 83.3% and specificity of 100%, indicating that circulating cfDNA sequencing contributed to an improvement in the diagnosis of PJP in patients with RD. For the three patients for whom plasma cfDNA sequencing failed to detect *P. jirovecii*, respiratory tract samples were used to detect PJP infection. Intriguingly, a higher level of β-D-glucan was found in patients with positive results for *P. jirovecii* in plasma cfDNA sequencing [230.1 pg/ml (IQR 168.4–639.5 pg/ml) *vs* 37.5 pg/ml (IQR 10.0–136.5pg/ml); $P = 0.017$].

Table 2 The demographic and clinical characteristics of patients with *Pneumocystis jirovecii* pneumonia and rheumatic disease

No.	Age	Gender	Underlying diseases	Disease duration (days)	LDH (U/l)	β-D-glucan (pg/ml)	*P. jirovecii* reads (plasma cfDNA)	*P. jirovecii* reads (respiratory specimen)	Outcome
1	65	Male	DM (anti-MDA5)	144	313	2666.4	12	–	Died
2	54	Male	DM (anti-MDA5)	81	413	10.0	0	Sputum 20996	Died
3	36	Female	DM (anti-MDA5)	123	650	104.8	37	–	Died
4	50	Female	DM (anti-MDA5)	37	237	37.5	–	BALF 66	Survived
5	52	Male	DM (anti-MDA5)	85	482	10.0	–	BALF 1167	Died
6	57	Female	DM (NA)	126	20 954	235.5	144	–	Died
7	68	Female	DM (anti-MDA5)	45	290	197.8	4	–	Survived
8	55	Male	DM (anti-MDA5)	90	861	769.5	1	–	Died
9	29	Female	SLE	119	521	1284.8	124	–	Survived
10	55	Female	SLE	7300	673	201.6	55	–	Survived
11	68	Female	AAV	153	577	663.2	–	Sputum 2	Died
12	84	Male	AAV	175	759	229.5	297	Sputum 58	Died
13	17	Female	AOSD	126	489	230.6	3	–	Survived
14	73	Female	AOSD	132	694	110.9	7	–	Survived
15	56	Male	AOSD	180	1657	136.5	0	BALF 482	Survived
16	44	Male	RA	18	393	37.5	0	Sputum 4	Died
17	52	Female	UCTD	1949	429	158.6	250	–	Died
18	39	Male	SLE	270	228	249.4	57	–	Survived

AAV: ANCA-associated vasculitis; anti-MDA5: anti-melanoma differentiation-associated gene 5 antibody; AOSD: adult-onset Still's disease; BALF: bronchoalveolar lavage fluid; cfDNA: cell-free DNA; LDH: lactate dehydrogenase; NA: not available; PJP: *Pneumocystis jirovecii* pneumonia; RD: rheumatic disease.

Discussion

PJP has emerged as a life-threatening disease in immunocompromised hosts, and late diagnosis is associated with higher mortality [4, 11]. Diagnosis has been more difficult owing to non-specific pulmonary manifestation, radiographic abnormalities and lower sensitivity of microscopy-based diagnostic tests in non-HIV-infected immunocompromised hosts [3]. PCR-based tests still lack standardization and validation [12]. As a modern diagnostic method, mNGS has shown potential in identifying PJP, as reported in several studies [13, 14]. In renal transplant recipients, blood samples are used with mNGS for the diagnosis and treatment guidance [15]. Patients with RD have common manifestations, such as fever, cough and dyspnoea, making it difficult to differentiate infections from active RD. In the present study, we addressed the functionality of plasma cfDNA sequencing using mNGS for the diagnosis of PJP.

In this study, eight patients with DM accounted for the highest proportion in the population with PJP, and seven patients carried anti-melanoma differentiation-associated gene 5 (anti-MDA5) antibody, which was mainly attributed to intensive immunosuppressive therapy and concomitant interstitial lung disease. A 13-year retrospective cohort study that reviewed 76 966 patients with systemic rheumatic diseases [16] concluded that the incidence rates of opportunistic infections were highest for PM/DM cases (61.3/1000 person-years). The early identification and treatment of infection is crucial to improve the prognosis.

Remarkably, 80% of patients with PJP showed positive results in plasma cfDNA sequencing. The small numbers of *P. jirovecii* reads with high proportions of fungi were of diagnostic value in our study, although the clinical characteristics of underlying conditions and fungal load differed across individuals with RD, which might affect the sensitivity of plasma cfDNA [17]. Currently, cfDNA is fairly reliable for pathogen identification from sterile body fluids in clinical use [7]. It has been reported that *P. jirovecii* in the human lungs could circulate into plasma, especially in immunocompromised patients in the event of PJP infection [18]. In a study by Wang *et al.* [19], serum cfDNA for quantitative PCR resulted in a detection sensitivity of 68.6% and specificity of 97.2% in the diagnosis of PJP in immunosuppressed individuals. Intriguingly, *P. jirovecii* sequences were not detected with plasma cfDNA sequencing from the control group in our study, showing the potential of circulating cfDNA sequencing to rule out PJP infection in RD patients.

An elevated serum β-D-glucan level is an adjunct for the diagnosis of probable PJP infection when excluding other invasive fungal diseases. In the present study, we demonstrated concordance between plasma cfDNA and β-D-glucan for the identification of *P. jirovecii*, which suggests that a positive β-D-glucan test result might be a good indicator to detect plasma cfDNA and confirm a diagnosis of PJP. In addition, we demonstrated that prophylaxis is important

when initiating glucocorticoid and immunosuppressant therapy when treating patients with RD [20], especially during the first 4 months post-diagnosis, when patients present generalized disease activity and are receiving strong immunosuppressive therapy. Hence, being aware of the risk of PJP after combining immunosuppressants when treating RD is crucial.

Clinical metagenomics have brought about a paradigm shift in aetiological diagnosis. Our study offers evidence for the value of circulating cfDNA detection using NGS to diagnose PJP in patients with RD, especially when respiratory samples are unavailable. Owing to the limited sample size, further studies are important to address the sensitivity and specificity of circulating microbial cfDNA for PJP identification in large cohorts of patients with RD.

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

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